7-31-2014 12:00 AM

Immune function and infection status is related to migration distance and phases of the annual cycle in song sparrows (Melospiza melodia)

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology
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Immune function and infection status is related to migration distance and phases of
the annual cycle in song sparrows (*Melospiza melodia*)

(Thesis format: Monograph)

by

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Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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Abstract

Life history theory predicts animals adjust immune investment based on their risk of encountering familiar and unfamiliar parasites. Although long-distance migrants may encounter a greater diversity of parasites than short-distance migrants, the energetic costs of migration may constrain immune investment. However, few studies have investigated the relationship between migration distance and immunity. I used stable isotope analysis to estimate the wintering latitude, and thus migration distance, of song sparrows (*Melospiza melodia*). I assessed whether migration distance was related to the incidence of infection with a blood-borne parasite, parasite load, and immune function. The incidence of infection increased with migration distance, but only for adults. Macrophage phagocytosis, associated with innate immunity, decreased with migration distance but only for males. As birds shifted from migratory to breeding condition, innate immunity decreased while acquired immunity tended to increase. These findings suggest that exposure to parasites varies with migration distance, the demands of migration may constrain immune investment, and innate immunity may be important during migration.

Keywords:

Annual cycle, acquired immunity, differential migration, ecological immunology, infection, innate immunity, life history, *Melospiza melodia*, migration, parasites, stable hydrogen isotopes.

Acknowledgments

I must first express my immense and sincere gratitude to my supervisor, Professor Beth MacDougall-Shackleton, and co-supervisor, Professor Scott MacDougall-Shackleton, for taking me under their 'wing'. Without their guidance, support, and encouragement completing this research would not have been possible. Seriously, thank you. I must also acknowledge the support of my advisors Professor Chris Guglielmo and Professor Jeremy McNeil, who provided great insight and guidance in this research. I thank Professor Gulglielmo for guidance and use of his lab equipment to clean toenail samples. I thank Jeremy McNeil for his advice on thesis writing and supporting timely execution of my thesis. I am very grateful to all the members of both Mac-Shack labs for helpful advice regarding my presentations and general life: Yani Sarquis-Adamson, Drew Moore, Natalie Westwood, Joel Slade, Ricki Lovett, Dominique Potvin, Shannon Mischler, Mélanie Guigueno, Tara Farrell, Andria Diez, Adam Piraino, Andrea Boyer, Michael Hasstedt, and Alannah Lymburner. In particular, I thank Heather MacGillivray for our extraordinary partnership in completing our data collection and lab work. I thank the staff in the University of Western Ontario, Biology Department who answered many questions regarding graduate studies at Western. I am grateful to staff and members of the Queen's University Biological Station and the Advanced Facility for Avian Research for creating a positive and effective research environment. I thank my mothers, Donna Kelly and Margaret Kelly, siblings, Kristi Benger, Stephen Benger, Stephanie Benger, Denys Kelly, Richard Kelly, and Ally Benger, and dearest friends Buddy and Nick Wilpstra for their encouragement and incredible support while completing my thesis. Above all I must thank my father, Peter Kelly, for his reassurance and "buckle-down-and-just-do-it" attitude. I love you too.

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1.1   Host-parasite interactions and vertebrate immunity

Parasites, defined as organisms residing in or on another organism (the host) which they exploit for resources (Schmid-Hempel, 2011), are widespread both taxonomically (representing about 40% of known species; Dobson et al., 2008), and geographically (occurring on all six continents; Schmid-Hempel, 2011). This means that few organisms avoid exposure to parasites during their lifetime and consequently have evolved a wide range of behavioural and physiological defences to help avoid, resist or control parasitic infection (Klasing, 2004; Demas and Nelson, 2012). In particular, both vertebrates and invertebrates have evolved immune systems that function to differentiate 'self' from potentially harmful 'non-self' substances and eliminate the latter (Demas et al., 2011). As a result, immunocompetence, or the ability to mount an appropriate immune response following exposure to a pathogen or parasite, is a critical component of fitness (Demas and Nelson, 2012).

Avian blood-borne parasites

Interactions between birds and their parasites have been well-studied from a variety of perspectives, such as sexual selection (Hamilton and Zuk, 1982), conservation (McCallum and Dobson, 2002) and human health (Prugnolle et al., 2011). Birds are infected by a wide variety of Haemosporidian and other blood-borne parasites transmitted by a variety of arthropod vectors. In particular, Haemosporidian parasites (blood-borne

protozoa belonging to family Apicomplexa and including genera *Plasmodium,*
Haemoproteus, and *Leucocytozoon*) infect 68 % of avian species and occur in every
continent except Antarctica (Atkinson and Van Riper, 1991). Haemosporidia rely on
arthropod vectors to move between hosts, with *Haemoproteus* and *Plasmodium*
transmitted by mosquitoes (Cox, 2010) and *Leucocytozoon* transmitted by mosquitoes
and blackflies (Atkinson and Van Riper, 1991). In addition to Haemosporidia, other
blood-borne parasites of birds include *Trypanosoma* (vectored by mites, louse flies,
biting midges, culicine mosquitoes, and blackflies; Vickerman, 1985) and microfilaria
(the larval stage of nematodes, vectored by blackflies, biting midges, horseflies and
mosquitoes; Ravinder et al., 2005).

Effects of blood-borne parasites on host fitness.

Parasites have negative effects on host fitness including reduction in hosts' sexual
ornamentation, reproductive success, and survival. For example, male yellowhammers
(*Emberiza citronella*) infected with *Haemoproteus* had less colourful plumage and
produced fewer fledglings than did uninfected males (Sundberg, 1995). Similarly, male
pied flycatchers (*Ficedula hypoleuca*) found to be infected with *Trypanosoma* produced
20 % fewer recruits than did uninfected males (Rätti et al., 1993). Female blue tits
(*Cyanistes caeruleus*) treated with antiparasitics that reduced *Haemoproteus* infection
had higher hatch success, provisioning rates, and fledging success than did control
females with chronic infections (Knowles et al., 2010), as well as higher overwinter
survivorship (Martínez-de la Puente et al., 2010).

Innate and acquired immunity

In response to such negative effects of parasites host species have evolved an immune system to prevent and/ or limit parasitic infections. The immune system is a complex assembly of specialized cells whose products protect an organism from infection as well as proliferation of harmful non-self constituents, such as parasites (Demas et al., 2011). Different aspects of vertebrate immunity are organized into 'branches' according to their roles (Figure 1.1) and the two main branches are innate and acquired immunity (Demas et al., 2011; Jacobs and Zuk, 2012).

Innate immunity serves as the initial defence against invading pathogens and/or parasites and consists of a standing array of rapid and nonspecific responses (Demas et al., 2011; Jacobs and Zuk, 2012). These include anatomical barriers (mucosal membranes and skin), humoral factors (lysozyme, complement, acute phase proteins), and cellular responses (phagocytic cells, inflammatory mediating cells, and natural killer cells) (Demas et al., 2011). An important component of innate immunity is the complement system which opsonizes pathogens and infected cells to promote inflammatory responses and lysis, as well as foreign cells for phagocytosis. The complement system serves as the intersection of innate and acquired immunity, as it is responsible for stimulating the acquired immune response by opsonizing potential threats for destruction (Dempsey and Fearon, 1996).

Acquired immunity defences require activation by the complement system and consequently is slower to respond than the innate immune response. Acquired immunity permits antigen-specific responses and creates a recognition system to prevent a second infection by a previously encountered pathogen and/or parasite (Demas et al., 2011).
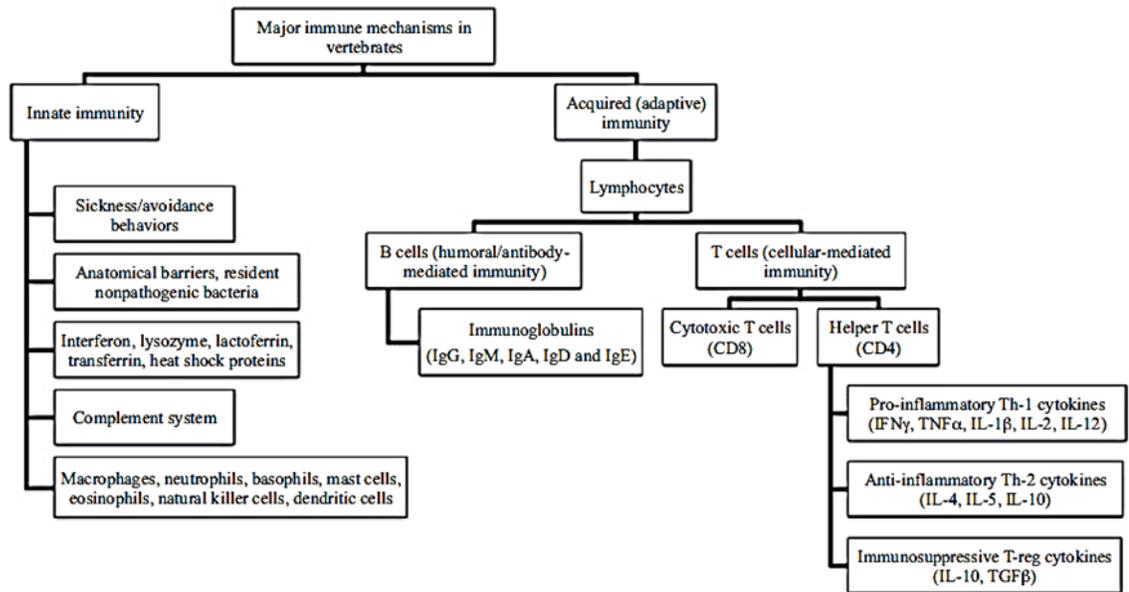
Figure 1.1 Major divisions of the vertebrate immune response. Adapted from Demas et al., 2011.

The acquired immune response can be further divided into two sub-branches: the *cell-mediated* response of T lymphocytes, and the *humoral* response of B lymphocytes. The latter produces antibodies responsible for the specificity and memory of the acquired immune response (Demas et al., 2011). Together, the innate and acquired immune systems defend against novel and familiar pathogens and parasites.

Quantifying immunocompetence

Immunocompetence is the ability of an individual to mount an appropriate immune response against a foreign pathogen or parasite (Demas et al., 2011; Zuk and Stoehr, 2002). Given the many different functions of the immune system, quantifying exactly what constitutes a strong immune system can be difficult. In the past, studies examining immune function of organisms were usually based on a single measure of delayed-type immune sensitivity (e.g., the phytohemagglutinin-induced swelling response) (Norris and Evans, 2000). While such assays are informative, using a single measure of immune function to draw general conclusions implicitly assumes that all branches and sub-branches of immunity are positively correlated but a growing body of work shows that different aspects of immunity may instead be unrelated or even negatively related (Martin et al., 2006; Martin et al., 2007; Buehler et al., 2008; Kubli and MacDougall-Shackleton, 2014). Thus, reliably assessing an organism's immunocompetence requires that researchers measure a range of immune functions (Demas et al., 2011).

Costs of immunity

Although critical to fitness, immune responses are costly, not only because of the metabolic requirements of immune cells, but also due to the indirect consequences of immune up-regulation. These include wasting lean tissue as a result of excessive gluconeogenesis during chronic infections to fuel immune cells as well as anorexia during a period when the body requires nutrients to support the demands of mounting an immune response (Scrimshaw, 1991; Lochmiller and Deerenberg, 2000).

Innate and acquired immunity follow different developmental schedules and, accordingly, differ in their energetic costs. The innate system is the first line of defence against an antigen and as such must be continually maintained incurring high energetic costs associated with constant maintenance of protective cells (phagocytes) and proteins (natural antibodies; Klasing, 2004). However, the non-specificity of innate immunity means it develops more quickly than an acquired immune response. An acquired immune response requires antibodies to recognize antigens; these antibodies are developed by semi-random recombination and point mutations in their coding genes (Klasing, 2004). Such diversification is necessary to allow the immune system to recognize diverse antigens but only rarely does a recombination event result in a functional antibody. This trial and error system to develop an acquired immune response involves a high energetic cost (Klasing, 2004). However, once an antibody is created for an antigen, the antibody remains in the system. In comparison, then, the costs of innate immunity arise from the energetic costs of continually maintaining defences, while the costs of acquired immunity arise from the long time needed to develop an appropriate antibody (Klasing, 2004).

Life history theory predicts that, because immune function is energetically costly, organisms face trade-offs between investing in immunity and in other energetically demanding activities (Lochmilller and Deerenberg, 2000; Norris and Evans, 2000), a main focus of the emerging field of ecological immunology. For example, when resources are limited they must be partitioned between immunity and reproductive success as avian reproduction is costly due to the energetic requirements of producing eggs and raising young. Supporting this is the negative relationship of immunity with reproductive effort in parental pied flycatchers (*Ficedula hypoleuca*; Moreno et al., 1999) and parental male barn swallows (*Hirundo rustica*; Saino et al., 2002). In both studies, parents of experimentally increased broods showed weaker T-cell mediated immunity while experimentally decreased broods showed enhanced T-cell mediated immunity.

The annual cycle and immunity

Migratory birds have many phases to their annual cycle: non-breeding, migration to breeding grounds, breeding, moult, and migration to non-breeding grounds. Each phase involves behavioural and physiological responses to seasonal changes in the environment, such as hormonal changes in the breeding phase of the annual cycle, which may modulate immune response (O'Neal and Ketterson, 2012). Testosterone, the primary male gonadal androgen, enhances expression of sex signals as well as affecting aggressive, reproductive, and parental behaviour (Ketterson and Nolan, 1994), and may also suppress immune function (Folstad and Karter, 1992). Male dark-eyed juncos (*Junco hyemalis*) exposed to prolonged elevation of testosterone exhibit suppressed antibody production and cell-mediated immunity (Casto et al., 2001), although other studies have

reported no effect of testosterone on immunity or immunoenhancing effects (reviewed in O'Neal and Ketterson, 2012). Immune function may also vary across phases of the annual cycle in relation to an individual's risk of encountering (and re-encountering) particular pathogens or parasites (Norris and Evans, 2000). For example, the prevalence of infection by *Plasmodium, Haemoproteus,* and *Leucocytozoon* in migratory garden warblers (*Sylvia borin*) was greatest on the breeding grounds and during the onset of autumn migration and was most scarce during spring and autumn migration (Hellgren et al., 2013).

Outside of the breeding phase of the annual cycle, immune function in birds is expected to be more robust because this period is free from the energetic and physiological demands imposed by reproduction (Martin et al., 2008). Moreover, birds may need to increase immunity outside of the breeding phase in order to prepare for harsh winter climates (Nelson and Demas, 1996). Supporting this idea, captive house sparrows (*Passer domesticus*) exhibit higher T-cell mediated immunity outside of the breeding phase than during early breeding (Martin et al., 2004). Importantly, for migratory bird species the breeding and non-breeding life-history phases of the annual cycle are connected by seasonal migrations that involve specific behavioural and physiological costs and adaptations (see below). Coordinating these behavioural and physiological adaptations is necessary to cope with seasonal energy shortages, and is thus a critical determinant of fitness (Nelson and Demas, 1996).

1.2 Avian Migration

Migration is present in all major branches of the animal kingdom and can vary from the daily movements of plankton in the water column to the long-distance, seasonal migrations of mammals, reptiles, fish, and birds (Dingle and Drake, 2007). The broad taxonomic, temporal, and geographic scale over which migration occurs means that defining migration depends on the perspective from which it is being explored (Dingle and Drake, 2007). In this thesis, which examines the annual migration of small passerine songbirds, migration is defined as the seasonal movement of individuals between breeding and wintering grounds (Dingle and Drake, 2007).

Across bird species, the distances travelled during their annual migration range from very long [approximately 70,900 km in Arctic terns (*Sterna paradisaea*; Egevang et al., 2010), up to 29,280 km in bar-tailed godwits (*Limosa lapponica*; Battley et al., 2012), and approximately 26,000 km in red knots (*Calidris canutus rufa*; Niles et al., 2010)] to relatively short [up to 40 km in the altitudinal migration of dark-eyed juncos (*Junco hyemalis hyemalis*; Rabenold and Rabenold, 1985), and up to 27 km in the altitudinal migration of satyr tragopans (*Tragopan satyra*; Norbu et al., 2013)]. Thus, although the act of migration is widespread among birds, the distance that individuals travel between the breeding and non-breeding grounds varies greatly across species.

Why migrate?

Three ecological factors are hypothesized to explain the evolution of bird migration: food limitation, direct climatic effects on physiological function, and risk of nest predation (Boyle and Conway, 2007). There may not be one single correct

hypothesis as to why animals migrate. Indeed, the hypotheses reviewed below are not mutually exclusive. For example, small-bodied individuals may migrate because suitable food resources are not available year-round in one location but also to avoid thermal extremes, and to reduce predation risk. The main ecological factors that promote migration likely differ among and within species, but the maintenance of migration in many bird species suggests that the benefits of migration do in many cases outweigh the risks and costs.

First, the food limitation hypothesis posits that animals whose diets are not seasonally reliable (e.g., fruit or nectar) migrate in order to gain access to a more abundant food source (Levey and Stiles, 1992; Boyle and Conway, 2007; Jahn et al., 2010). In support of this, fruit production is highly seasonal and frugivory is associated with increased likelihood of migrating (Levey and Stiles, 1992). In addition, when fruit productivity ceases at low altitudes, white-ruffed manakins (*Corapipo altera*) make seasonal migrations uphill to exploit greater abundances of fruit at found at higher altitudes (Boyle, 2010). Similarly, insect abundance is also seasonal, and insectivory is associated with increased migratory distance (Levey and Stiles, 1992; Boyle and Conway, 2007).

Second, the climatic effects hypothesis posits that seasonal changes in temperature or other climate factors can induce migration if abiotic conditions indicate an individual cannot survive in that area (Ketterson and Nolan, 1976; Jahn et al., 2010). Two variants of the hypothesis are (1) the thermal tolerance hypothesis, which suggests that migratory individuals are those who cannot endure thermal extremes, e.g. those with unfavourable surface area to volume ratios; and (2) the fasting endurance hypothesis,

which predicts that individuals at a greater risk of starvation (i.e. small individuals) will migrate (Ketterson and Nolan, 1976; Cristol et al., 1999). Consistent with the former, the slate-coloured junco (*Junco hyemalis*) nests farther at lower latitudes than the white-crowned sparrow (*Zonotrichia leucophrys*), white-throated sparrow (*Z. albicollis*), and tree sparrow (*Passer montanus*) and likewise has a greater tolerance to heat (Kendeigh, 1945).

Third, migratory movement may be favoured if there is latitudinal or altitudinal variation in nest predation and moving to the breeding grounds enables migrants to reduce nest failure; this is known as the nest-predation hypothesis (Boyle and Conway, 2007). For example, the nest predation of field sparrows (*Spizella pusilla*) increases with the density of the breeding population so they will migrate from a high-density wintering area to a low-density breeding habitat (Fretwell, 1968). The dickcissel (*Spiza americana*) also suffers high nest predation in areas with high nest densities and may therefore have gained increased reproductive success by migrating to areas of low breeding densities (Zimmerman, 1982; Cox, 1985).

Intraspecific variation in avian migration

Just as there are interspecific differences in the migratory tendency and distances, similar intraspecific differences can exist. For example, in blue tits (*Parus caeruleus*), females are more likely to migrate than males, while juveniles are more likely to migrate than adults (Smith and Nilsson, 1987). Species in which some, but not all, individuals migrate are termed partial migrants. Chapman et al. (2011) identify three main types of partial migration:

1. Non-breeding partial migration: All individuals breed in the same region but some individuals migrate in the non-breeding season while others remain on the breeding grounds.

2. Breeding partial migration: Individuals breed in different regions but overwinter in the same location.

3. Skipped breeding partial migration: While individuals must migrate to breed, some individuals do not do so every year, and remain on the non-breeding grounds.

Most research has focused on non-breeding partial migration, as in the case of blue tits mentioned above.

Similar to partial migration is differential migration in which all individuals migrate between the breeding and non-breeding grounds but individuals vary predictably from one another in the distance travelled or timing of migration typically according to age or sex classes (Ketterson and Nolan, 1985; Terrill and Able, 1988). Thus, partial migration describes the situation where some individuals remain sedentary whereas in differential migration all individuals migrate but migration distance varies. For example, all snow buntings (*Plectrophenax nivalis*; Banks et al., 2009) and red-winged blackbirds (*Agelaius phoeniceus*; Cristol et al., 1999) migrate but females make longer migrations than males.

Causes of partial and differential migration

Generally, if all individuals of a species or of a population are seasonal migrants, the benefits of migration should consistently outweigh the costs. Likewise, if some but not all individuals migrate, the relative advantages of migrating versus remaining year-round on the breeding grounds may vary across individuals and across age and sex classes (Ketterson and Nolan, 1985). Three hypotheses have been suggested to explain age or sex differences in migration and migration distance: (1) the body size hypothesis, (2) the dominance hypothesis and (3) the arrival-time hypothesis. No single hypothesis reviewed below is likely sufficient to explain partial and differential migration across all species. For example, blue tit migration supports the dominance hypothesis (Smith and Nilsson, 1987) while house finch migration does not (*Carpodacus mexicanus*; Belthoff and Gauthreaux, 1991). Even for a given species, these hypotheses are not mutually exclusive as willow tit migrations are consistent with the arrival-time hypothesis but may also support the body size hypothesis if there are thermal benefits to group formation (Chapman et al., 2011).

The body size hypothesis suggests that differential or partial migration may be related to variation in ability to endure severe conditions as a function of mass (Ketterson and Nolan, 1976). For example, male house finches (*Carpodacus mexicanus*) are larger and winter farther at higher latitudes than do females (Belthoff and Gauthreaux, 1991). Likewise, male dark-eyed juncos are larger, have greater fasting endurances, and also winter at higher latitudes than females (Ketterson and Nolan, 1976). Additionally, male tropical kingbirds (*Tyrannus melancholicus*) are larger, have a greater energetic demand,

and migrate to wetter regions during the non-breeding phase of the annual cycle while smaller-bodied adult females reside at the drier breeding grounds (Jahn et al., 2010).

The dominance hypothesis posits that variation in social dominance drive age- and/or sex-related migratory tendencies and migration distance (Ketterson and Nolan 1976). This hypothesis makes three assumptions. First, age classes or sexes differ in resource-holding ability, so that members of one class limit members of another's access to resources and force the latter to depart. Second, the likelihood of migration or distance travelled is correlated with social rank such that lower-ranking individuals migrate farther. Last, as migration is costly, dominant individuals avoid migrating (Cristol et al., 1999). This hypothesis, is supported by the migrations of great tits (*Parus major*) where socially dominant males are less likely to migrate than females; while subordinate juveniles are more likely to migrate than all adults (Smith and Nilsson, 1987). However, socially dominant female house finches make longer migrations than males because their small body size cannot withstand cold environments (Belthoff and Gauthreaux, 1991).

Last, the arrival-time hypothesis proposes that whichever population class establishes and defends breeding territories benefits most from migrating shorter distances, as this allows them to return to breeding grounds early enough to obtain desirable territories and, consequently, breeding opportunities (Ketterson and Nolan, 1976). This may be because the costs of late arrival may be greater for the sex responsible for territory defence (failure to breed) than the other (delay in breeding; Cristol, 1995; Cristol et al., 1999). The arrival-time hypothesis assumes that all individuals or population classes leave their wintering grounds at the same time and migrate at the same pace (Cristol et al., 1999). This hypothesis is supported in the

migration of spotted sandpipers (*Actitis macularia*) where females compete for breeding territories, make shorter migrations, and also arrive earlier than males on the breeding grounds (Oring and Lank, 1982). The hypothesis may also be supported by the migration of red-winged blackbirds (*Agelaius phoeniceus*) where males arrive at and begin competing for breeding territories more than a month before females arrive (Cristol, 1995; Cristol et al., 1999).

Maintaining intraspecific variation

The widespread existence of intraspecific variation in migration distance and tendency raises the question of how such variation is maintained. Two hypotheses have been proposed to explain how selection might maintain within-variation in migration distance and migration tendency.

First, annual variation in winter severity may maintain variation in migratory behavior if migratory individuals have a greater probability of survival over non-migrants in relatively severe winters and non-migratory individuals have greater survivorship over migrants in years with moderate winters (Lack, 1968). It is also possible that migrants and non-migrants have equal survivorship within in a single year if non-migrants have low survivorship in the winter and migrants have low survivorship during migration, as observed in dark-eyed juncos (Ketterson and Nolan, 1985). Thus, different migration strategies may be associated with similar fitness tradeoffs.

Alternatively, variation in migration could be maintained if non-migratory individuals have greater winter mortality but survivors have greater reproductive success, mediated by competitive ability during the breeding season, than their migratory

conspecifics (Von Haartman, 1968). For example, migrant dark-eyed juncos have lower annual productivity than resident/ short-distance migrants but are also more likely to survive winter (Ketterson and Nolan, 1985). Thus, the costs (non-migrants: poor winter survivorship; migrants: poor reproductive success) and benefits (non-migrants: greater reproductive success; migrants: high winter survivorship) of each migration strategy are balanced.

Costs of migration

Of all phases of the annual cycle, those involving migration are arguably the most energetically demanding (Wikelski et al., 2003). The energetic cost of flight varies among and within species, due to differences in a bird's mass, mode of flight (e.g., soaring vs. flapping), and temperature of the air (Schmidt-Nielsen, 1972; Hedenstrom, 1993; Wikelski et al., 2003); regardless, the minimum oxygen consumption associated with flight has been found to be over twice that of running (Butler et al., 1998). Moreover, migratory birds routinely fly for extended distances, totaling upwards of thousands of kilometers, to cross oceans or other barriers. For example, bar-tailed godwits travel 29,000 km in only 20 days (Battley et al., 2012); arctic terns travel up to 670 km in a single day (Egevang et al., 2010); Swainson's thrush (*Catharus ustulatus*) and hermit thrush (*C. guttatus*) fly up to 600 km in a single flight (Wikelski et al., 2003); and great snipes (*Gallinago media*) fly up to 6,800 km in three days (Klaassen et al., 2011). During their journeys, birds cannot reduce energy costs by travelling more slowly, unlike running mammals, as the relationship between power and speed is U-shaped and the cost of flying is minimized at an intermediate speed (Alerstam and Hedenström,

1998). Furthermore, migrating birds are heavily burdened with fat which they use to fuel their long flights incurring unique energetic challenges (McWilliams et al., 2004).

Migration is also costly in terms of mortality and reproductive success. The incidence of mortality is higher during migration than overwintering or breeding as seen in black-throated blue warblers (*Dendroica caerulescens*) where the incidence of mortality during migration is 15 times higher than during stationary periods (Sillett and Holmes, 2002). Additionally, mortality is dependent of migration distance as seen with dark-eyed juncos (Ketterson and Nolan, 1985). Migration may also negatively affect reproduction due to their adjacent timing. For example, male white-ruffed manakins (*Corapipo altea*) that migrate have less mating success the following breeding season (Boyle et al., 2011). Additionally, migrant dark-eyed juncos suffer lower annual productivity (2.63 young fledged) than their resident counterparts (5.14 young fledged) (Ketterson and Nolan, 1985).

Evaluating avian migration distance

Until recently, characterizing individual variation in migratory tendency or distance was very difficult and relied on a small number of recaptures of banded individuals on the wintering grounds. Fortunately, the seasonal movements of migratory animals are now easier to study thanks to the advent of stable isotope analysis. Birds have metabolically inert tissues, such as keratinous feathers and claw tissues, which contain isotopic signatures reflecting those of the local food webs at the location they were formed (Michener and Schell, 1994). Thus, tissue can be sampled once to provide information as to where those tissues were grown, and thus where the individual was, in a

previous season (Hobson and Wassenaar, 1996), making it a cost-effective method to examine the migratory origins of birds. In North America, stable hydrogen isotopes (δD) of precipitation follow a strong latitudinal gradient (Figure 1.2), such that δD increases with decreasing latitude (Meehan et al., 2004). Thus, for birds completing northerly – southerly migrations, δD of claw or feather tissue can be used to estimate wintering latitude and thus, migration distance (Kelly et al., 2002).

An important caveat to my use of hydrogen isotopes to infer migration distance concerns the issue of fractionation, whereby a chemical process alters the stable isotope ratios because of slight chemical differences arising from subtle differences in mass between isotopes of hydrogen (Wassenaar, 2008). This creates a difference in isotopic signatures between δD of water and of an individual's diet, and then between diet and tissue. These cumulative differences, termed net isotopic discrimination, introduce error when estimating the latitude at which tissue samples were grown (Wassenaar, 2008). Although species differences in diet prevent identifying a single general discrimination factor applicable to all species, a discrimination factor of -25 ‰ has been suggested for songbird feather tissues (Hobson, 2008) and such a correction means birds are wintering farther at lower latitudes than their keratinized tissue suggests. Thus, my study uses δD signatures to compare only relative, not absolute, migration distances, and does not attempt to pinpoint the specific locations at which individuals overwintered. Importantly, because net isotopic discrimination should be similar across all samples (Wassenaaar, 2008) fractionation should not systematically bias my results.

The best way to overcome fractionation error is to generate tissue basemaps rather than comparing isotope signatures of tissues directly to that of precipitation. For my
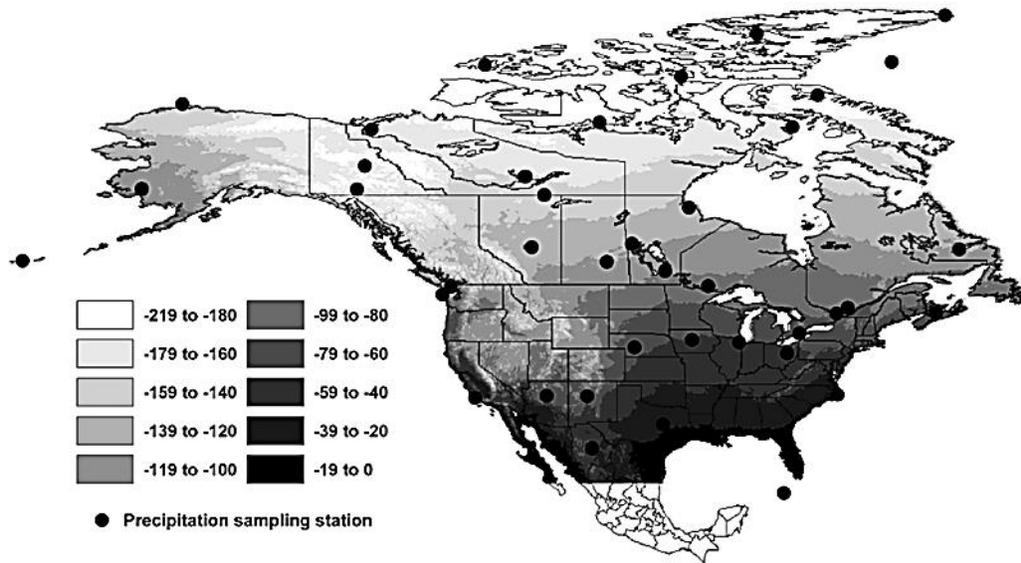
Figure 1.2 Altitude-corrected annual δD (‰) of North American precipitation. Circles are δD sampling stations used in map building. Adapted from Meehan et al., 2004.

work, this would involve sampling claw tissue from song sparrows throughout eastern

North America and creating a keratin basemap for δD. A more feasible way to overcome

fractionation error would be to create likelihood-based assignments from isotope data

using birds with known wintering locations (based on band recoveries or geologger data;

Van Wilgenberg and Hobson, 2011).

Migration and parasite risk

Although the energetic and survival costs associated with migration itself have

been well studied, less attention has focused on the costs imposed by parasites found in

different seasonal habitats. Migratory birds presumably encounter more diverse parasite

fauna (Møller and Erritzøe, 1998) as parasites vary in diversity and prevalence at

different geographical scales (Dobson et al., 2008; Merino et al., 2008). For example, the

prevalence of *Haemoproteus columbae* varies among five nearby populations of feral

pigeons (*Columba livia*; >25 km apart, but <50 km), apparently due to the abundance of

the louse fly vector (Sol et al., 2000) and the prevalence of *Haemoproteus* and

Plasmodium in 26 Chilean bird species varies with latitude (Merino et al., 2008). In

addition to geographic differences in parasite richness and abundance, the prevalence of

different parasite lineages also varies geographically, as seen in *Plasmodium* lineages of

common yellowthroats (*Geothlypis trichas*; Pagenkopp et al., 2008).

Host species with larger geographic ranges have greater species diversity of

parasites (Price et al., 1988). Geographic variation in the prevalence and diversity of

parasite communities means that the risk of parasitism will be greater for migratory

individuals who occupy more than one geographic area during their lifetime and the

specific parasites to which a potential host is exposed will vary according to migratory tendency (Møller and Erritzøe, 1998). For example, the incidence of infection by Haematozoan parasites increases with migration distance across waterfowl species (Figuerola and Green, 2000). Additionally, most birds leave breeding grounds to overwinter closer to the equator where there is higher parasite abundance (Merino et al., 2008) and species richness (reviewed by Rohde, 1992), resulting in higher prevalence of *Haemoproteus* and *Plasmodium* infections near the equator (Merino et al., 2008).

Migration and immunity

Given the increased abundance and diversity of parasites that migrants may encounter, together with the negative effects of parasites on host fitness, one might predict based on life history theory that migrants would invest more in immune defence than non-migrants. Indeed, migratory bird species have proportionately larger immune-related organs (bursa of Fabricius and spleen) than closely related non-migratory species (Møller and Erritzøe, 1998). However, migrants may invest less in immunity given the energetic demands of flight and the need to catabolize lean tissue to fuel migration (McWilliams et al., 2004) especially during or immediately after migration. For example, Swainson's thrushes (*Catharus ustulatus*) reared under conditions favouring migration have lower T-cell mediated immunity compared to control birds (Owen and Moore, 2008a) and European starlings (*Sturnus vulgaris*) show decreases in innate immune function after completing endurance flights (Nebel et al., 2012). Thus, although migratory birds may encounter more parasites and thus stand to benefit from investing in

immunity, their ability to do so may be constrained by the energetic demands of migration.

As reviewed above, optimal immune allocation should vary with the diversity and abundance of parasites that a host is likely to encounter, as well as with other competing demands such as migration. Moreover, the optimal balance between the innate and acquired branches of the immune system should depend upon the risk of encountering novel versus familiar parasites so "slow-living" species (long lifespan, long development times, many small clutches) would be expected to invest more in acquired immunity which can 'remember' previously encountered parasites due to their high likelihood of re-encountering the same parasites, whereas "fast-living" (short lifespan, short development times, few large clutches) species are unlikely to repeatedly encounter the same parasites and should thus rely more on innate defences (Ricklefs and Wikelski, 2002; Martin et al., 2007). In support of these predictions, complement activity (innate immunity) is positively correlated with clutch size across tropical bird species (Lee et al., 2008). Additionally, the secondary antibody response of house sparrows (*Passer domesticus*) to a novel antigen is greater in "slow-living" versus "fast-living" individuals of a population (Martin et al., 2006). This logic can be extended to make predictions about immune allocation in birds that vary in migration behaviours. Migrants (especially long-distance migrants) should benefit by investing more in innate immunity than non-migrants, because they may encounter a more diverse set of parasite fauna over their lifetimes. Conversely, non-migrants (and to a lesser extent short-distance migrants) may benefit more from investing in acquired immunity as they are more likely to encounter the same parasites repeatedly. However, little empirical work has examined the relationship

between immune investment and migration distance among species (Mendes et al., 2006),

and remains entirely unexplored at the within-species level.

1.3 Objectives and Hypotheses

In this thesis I used stable isotopes to infer the wintering latitude, and thus the

migration distance, of individual song sparrows (*Melospiza melodia*) captured at their

summer breeding grounds. I determined if there is a relationship between an individual's

migration distance and (1) the incidence and intensity of infection by blood-borne

parasites, and (2) measures of innate and acquired immune function. Finally, I examined

seasonal patterns of parasitism and immune function, as song sparrows transition from

the spring post-migration phase through the breeding phase. Exploring these parameters

will improve our understanding of how birds may adjust immunity in response to

familiarity of parasites as well as how birds balance the demands of migration and

immunity. Such understanding is important considering the concern over migratory birds

as long-distance transporters of disease.

Study system

The Eastern song sparrow, *Melospiza melodia melodia*, is a good model to

examine individual variation in migration distance, parasitism and immune allocation as

their migration distance ranges from 250 km to 1500 km and those overwintering in

Ontario spend the winter at the lowest latitudes (Figure 1.3; Davis and Arcese, 1999).

Song sparrows breeding and banded near Newboro, Ontario, have been recaptured during

winter in Tennessee and Maryland, but local farmers at Newboro, ON have also reported sightings of banded birds (presumably from the same breeding population) at the study site during winter months (S. A. MacDougall-Shackleton, unpublished data). Thus, this breeding population varies significantly with respect to migration distance and potentially whether some individuals migrate at all. Moreover, song sparrows breeding at the study site are infected by a variety of Haemosporidian and other blood-borne parasites, some of which are known to vary geographically (e.g. *Plasmodium* lineages; Pagenkopp et al., 2008).

Hypotheses and predictions

The main objective of my thesis is to determine how within-species variation in migration distance relates to the incidence and diversity of parasitism as well as immune function. I captured song sparrows on the breeding grounds and determined the stable hydrogen isotope signature (δD) of claw tissue grown the previous winter, in order to estimate wintering location (a proxy for migration distance).

Song sparrows vary substantially in migration distance, based on previous band-recapture evidence for this species (Nice, 1937; Davis and Arcese, 1999). Some of this variation may be associated with sex differences as many songbirds, including male song sparrows, establish and defend territories (Nice, 1937; 1942) and may thus benefit by arriving earlier. Furthermore, adult song sparrows are dominant to juveniles and, as a result, young-of-the-year may migrate farther than adults to avoid competition for resources (Arcese and Smith, 1985). Considering these traits, females and young-of-the-year will make the longest migrations and adult males to make the shortest migrations.
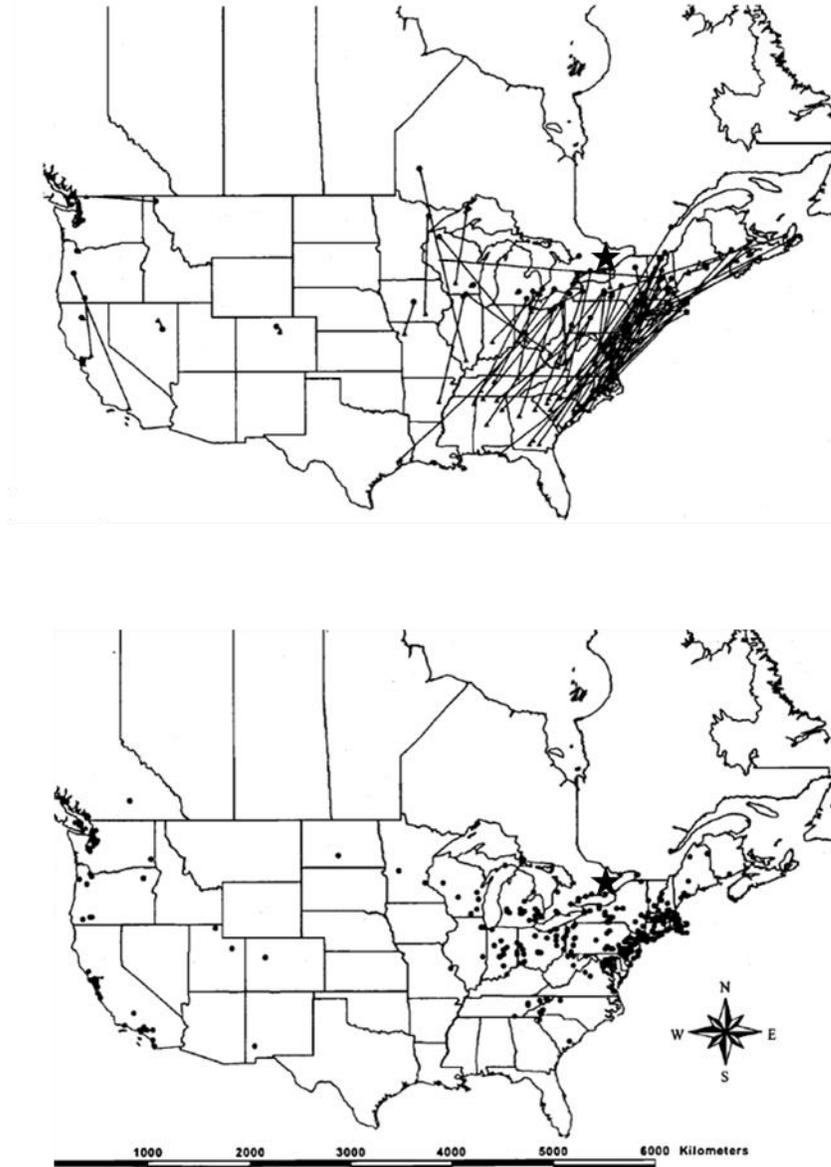
Figure 1.3 Generalized migration routes by song sparrows (top) between breeding (circle) and wintering (triangle) locations, based on banding and recovery data in North America Locations of resident song sparrows (bottom) with banding and recovery locations within the same area for breeding and wintering banding and recovery Both figures adapted from Davis and Arcese, 1999. Stars indicates location of field site near Newboro, Ontario, Canada.

Similar patterns are seen in other partially and/ or differentially migrant species (e.g. dark-eyed juncos, Ketterson and Nolan, 1985; blue tits, Smith and Nilsson, 1987; house finches, Belthoff and Gauthreaux, 1991).

I will then determine whether migration distance is related to the incidence or intensity of infection with blood-borne parasites. I hypothesize that short and long-distance migrants (or migrants and non-migrants) will differ in the incidence of being parasitized and in their parasite load. Long-distance migrants presumably encounter a greater diversity and number of parasites than short-distance migrants and non-migrants (Møller and Erritzøe, 1998). I predict, then, the incidence of infection will increase with migration distance (seen across species; Figuerola and Green, 2000). Similarly, I predict parasite load will also increase with migration distance if the high energetic costs associated with migration reduce body condition and, in turn, ability to control infection once infected (Owen and Moore, 2008b).

I also hypothesize that long- versus short-distance migrants (or migrants versus non-migrants) will differ in their relative innate and acquired immune function. If activating one component of the immune response compromises the ability to invest in another immune component, organisms should strategically allocate resources to immune function based on their risk of encountering (and re-encountering) particular parasites (Norris and Evans, 2000). Because innate immunity is the first line of defence against unfamiliar parasites, and long-distance migrants may encounter a more diverse parasite fauna than short-distance migrants, I predict that innate immunity will increase with migration distance. Conversely, non-migrants or short-distance migrants are at greater risk of re-encountering familiar parasites and may thus rely more on acquired immune

defences; thus I predict that acquired immunity will decrease with migration distance. Alternatively, however, the high energetic demands of migration may constrain long-distance migrants from investing in either innate or acquired immunity; if so, both innate and acquired immunity may decrease with migration distance.

I also hypothesize that both the incidence of parasitism, parasite load, and immune function varies across the phases of the annual cycle, specifically as birds recover from migration and transition into the breeding phase. Rates of infection by blood-borne parasites should differ in birds captured early (recovering from migration) versus late (breeding phase) in the sampling period, due in part to seasonal increases in insect vector abundance (Møller, 2010). Moreover, the costs and benefits of investing in innate versus acquired immunity are likely to vary over the annual cycle due to changes in parasite abundance and familiarity. During migration, birds are less likely to re-encounter familiar parasites than when on their breeding or wintering grounds as they rarely stay in the same place for extended periods of time [stopover durations: 1-2 days in European robins (*Erithacus rubecula*; Bolshakov et al., 2007); 12 days for reed warblers (*Acrocephalus scirpaceus*; Schaub et al., 2001); 7 days for reed buntings (*Emberiza schoeniclus*; Schaub et al., 2001)]. Furthermore, the more time a bird spends on the breeding grounds, the greater its risk of re-encountering a familiar parasites and acquired immune defences become more valuable. Consequently, I predict that innate immune function will decrease, and acquired immune function will increase, throughout the sampling period.

2   **Materials and methods**

2.1 Study site, animals, and sample collection

I conducted my study using a colour-banded population of eastern song sparrows breeding near Newboro, Ontario (44.66 ˚N/76.22 ˚W) on land owned by the Queen's University Biological Station. This population has been studied for over ten years, thus age and breeding history is known for most individuals and the general field methods already established. Between April 15 and May 15, 2013, together with another member of the research team, I captured 56 adult song sparrows in seed-baited Potter traps. Traps were checked at least every 60 minutes, between 6:30 and 9:30 a.m. I determined the sex of each bird (N = 28 males, 27 females) based on the presence or absence of a cloacal protruberance (males) or brood patch (incubating females). I measured tarsus and wing chord length to the nearest 0.1 mm using dial calipers, measured mass to the nearest 0.2 g with a spring scale, and, if not already banded, I banded each bird with a unique combination of coloured leg bands for individual identification. I inferred age from previous years' banding records, and considered previously unbanded birds to be one year of age at first capture (Lapierre et al., 2011).

I used sharp scissors to clip two small toenail (claw) tissue samples (approx. 2.5 mm from each hallux toe) from each bird. To ensure that the tissue collected had been grown on the wintering grounds I performed a pilot study prior to the field season in which I measured hallux claw growth rate for 9 captive male white-throated sparrows (*Zonotrichia albicollis*). White-throated sparrows are closely related to song sparrows and like song sparrows, are ground foragers thus claw tissue should grow at a similar rate

(Mazerolle and Hobson, 2005). Liquid paper was applied to the base of the hallux claw and the distance from the base of the claw to the liquid paper was measured after two weeks of growth. The hallux claw grew at an average rate of 0.0317 mm ± 0.0031 mm (SE) per day so tissue deposited into the proximal end of the claw should take 223 ± 20 days to reach the distal end (claw tip). Accordingly, the entire claw (7 mm ± 0.29 mm) of a bird caught in late May should contain tissue growth from approximately the previous November (distal end) until the present (proximal end), while the 2.5 mm at the distal end should contain tissue grown between early November and late February. Song sparrows in my study population leave the breeding grounds in late July and do not return until early April (personal observation E. A. and S. A. MacDougall-Shackleton) and so the distal 2.5 mm of hallux tissue should reflect the isotopic signature of the bird's wintering location. I stored toenail samples at room temperature awaiting stable isotope analysis (see below). In addition to the 56 toenail samples from birds in 2013, I also had access to 47 samples (N = 32 males, 15 females) collected in spring 2012 using the same methods. Thirteen birds had toenail tissue collected in both 2012 and 2013.

Within 10 minutes of capture, I collected a blood sample of approximately 200 µL via brachial venipuncture, using sterile techniques (Millet et al., 2007). This involved removing feathers from the puncture site, followed by two wipes with an alcohol swab to remove dander and sterilize the area. Immediately after collection, I prepared a thin-film blood smear by placing a drop of whole blood onto a clean glass microscope slide, and used a second slide to gently spread the blood along the length of the first slide. Slides were allowed to air-dry, then were fixed in 100% methanol for 1 minute and stored at ambient temperature awaiting staining and microscopic analysis (see below). A portion of

the remaining whole blood sample was used for a phagocytosis assay (see below), performed in the field no later than 45 minutes after blood collection.

I kept the remaining whole blood at approximately 37 ˚C for no more than 3 hours before being spun for 10 minutes in a micro-hematocrit centrifuge at 13,000 rpm. I recorded hematocrit as the volume percentage of packed cells, and then collected plasma using a Hamilton syringe. Plasma was frozen and stored for no more than 3 months at -20 °C in labelled microcentrifuge tubes equipped with O-rings to prevent evaporation, until used in hemolysis-hemagglutination and immunoglobulin-Y assays (see below).

2.2 Immune assays

Phagocytosis assay

This assay infers the phagocytic activity of macrophages in whole blood, an aspect of cellular innate immunity, by quantifying the proportion of phagocytic cells that phagocytized at least one fluorescently labelled particle (Millet et al., 2007). I followed general procedures outlined by Millet et al. (2007), except that blood and reagent quantities were reduced following Kubli and MacDougall-Shackleton (2014).

Two weeks prior to the field season, I reconstituted Bioparticles ® of *Escherichia coli* (Invitrogen E-2864) fluorescently labelled with BODIPY ® FL 505/513 (Molecular Probes) to 20 mg/mL in tissue-grade phosphate-buffered saline (PBS) plus 2 mM sodium azide. This solution was kept at 4 °C and protected from light. Upon capture of a song sparrow, I prepared a dilution of Bioparticles into fresh, sterile, cell media [CO_2-independent media (Invitrogen 18045-088) with 4 mM L-Glutamine (Sigma-Aldrich

G7513), 5% Fetal Bovine Serum (FBS; Invitrogen 10437-010) and 1% penicillin-streptomycin (Invitrogen 15140-122)]. This dilution consisted of 2.25 µL reconstituted Bioparticles added to 680 µL cell media, to achieve a target 1:100 ratio of white blood cells to Bioparticles (Millet et al., 2007; Kubli and MacDougall-Shackleton, 2014).

Within 40 minutes of blood collection, I diluted 10 µL of whole blood 1:20 in fresh, 4 °C cell media. All pipetting and plating steps were carried out in a Plexiglass dead-air box, equipped with a HEPA filtration system, to ensure sterility (Kubli and MacDougall-Shackleton, 2014). I added 20 µL of the blood-cell media dilution together with 75.75 µL of diluted Bioparticles to each of 4 wells of an 8-well chamber slide (Sigma-Aldrich C7182). The slide was then covered and incubated at 41 °C for 15 minutes. Immediately after incubation, the slide was placed on ice for 5 minutes to end phagocytosis and gently washed twice with 91 µL of cold (4 °C) cell media. Adherent cells were fixed using a 91 µL wash of 100% methanol, and the slide was placed in a lightproof container for several months awaiting examination. For full protocol, see Appendix A.

In September 2013 I examined slides under a 60x objective using a fluorescent microscope (Leica DMLB) with an excitation/absorption spectrum of 505/513 nm and scored for the proportion of adherent cells (mainly macrophages) that had engulfed at least one fluorescent Bioparticle. 100 cells were scored per well, for a total of 400 cells scored per individual.

Hemolysis and hemagglutination assay (HL/HA)

This assay was conducted following previously published protocols (Matson et al., 2005) and quantifies two interrelated humoral components of innate immunity: natural antibodies (NAbs) and complement. NAbs are recognition molecules that opsonize invading microorganisms and initiate the complement enzyme cascade (Matson et al., 2005). Hemagglutination reflects the interaction between NAbs and antigens on foreign red blood cells, which results in blood clumping, whereas hemolysis reflects plasma levels of lytic complement proteins as well as NAbs, resulting in complement-mediated cell lysis (Mendes et al., 2006).

I performed HL/HA assays on thawed plasma during June and July 2013, using a 96-well plate format (clear, round-bottomed plates: Corning #3788). I added 25 µL of chicken plasma (Sigma #A9046) to columns 1 and 2 of plate rows A and H to serve as a positive control. Plate rows B through G contained plasma from my study subjects, using one row per bird. I added 25 µL of song sparrow plasma to columns 1 and 2 of these rows, and added 25 µL of PBS to columns 2 through 12 of all rows. For each row I then serially diluted the contents of column 2 through to column 11, such that plasma dilutions ranged from 1/2 (column 2) through 1/1024 (column 11). For each row, the column 12 well contained 25 µL of PBS and no plasma, and thus served as a negative control

After diluting the plasma samples, I added 25 µL of 1% rabbit red blood cell suspension (RRBC; Cedarland Laboratories, RBA 050; see Appendix for dilution protocol) to all wells. Plates were sealed with Parafilm, covered with a plate lid to minimize evaporation, and incubated at 37 °C for 90 minutes. After incubation, I tilted the long axis of the plate to a 45° angle and allowed it to sit for 20 minutes at room

temperature, then scanned the plate to score for aggllutination using a top-lift flatbed

scanner (EPSON Perfection 4990 Photo). I then kept plates lying flat at room temperature

for an additional 70 minutes, and then scanned again for lytic ability. Scoring for both

lysis and agglutination was done blind with respect to bird identity and migratory

distance. For full protocol including scoring technique, see Appendix B.

ELISA immunoglobulin Y (IgY) assay

I evaluated the circulating concentration of IgY in the blood as a measure of

acquired immunity. Immunoglobulins are produced by B cells and serve to recognize

specific antigens which in turn allows for long-lasting recognition of a specific antigen.

Specifically, IgY is the equivalent of IgG found in mammals and is most commonly

measured due to its high blood concentrations (Demas et al., 2011). IgY serves as the

major defence mechanism against systemic infections and has the ability to mediate

anaphylactic reactions (Demas et al., 2011; Warr et al., 1995).

I conducted IgY assays in June and July 2013. First, I adapted published protocols

(Bourgeon et al., 2006, Bourgeon and Raclot, 2006) for use in song sparrows by

determining the appropriate dilution of plasma to use. For this pilot study, I diluted 12

song sparrow plasma samples to a concentration of 1/500 (2 µL plasma to 1000 µL

dilution solution; see Appendix C for full protocol including solution recipes). Next I

added 200 µL of the diluted plasma to row A of columns 1 through 12 of a 96-well flat

bottom plate (Corning #3596), so that each column contained a unique song sparrow

plasma sample. I then filled rows B to H with 100 µL dilution solution. I performed serial

dilutions of each column by pipetting 100 µL out of row A and into row B. Contents of

the well were mixed well and then pipetted100 µL out of row B to row C, and continued this process to row H. Thus, final dilutions of plasma ranged from 1/500 in row A through 1/64000 in row H.

I covered the plate and incubated it for 1 h at 37 °C, then overnight at 4 °C. The next morning, I washed the plate twice with 200 µL of PBS-Tween solution then added 100 µL of 5% powdered milk solution to each well. After a second incubation at 37 °C for 1 hour, I washed the plate again twice with PBS-Tween, added 100 µL of anti-chicken IgY (diluted 1:250 in PBS-Tween; Sigma A9046), and incubated plates for 2 hours at 37 °C. After 2 more washes, I filled each well with 100 µL of revealing solution (31% hydrogen peroxide diluted 1:1000 in ABTS [2-2'azino-bis (3-ethyl-benzthiazoline-6-sulphonic acid)]) and incubated the plate once more for 1 hour at 37 °C. Immediately after this final incubation, I used a microplate reader (BIO-RAD iMark) to read plate absorbance using a 405 nm wavelength filter. IgY levels were expressed in units of absorbance. I then calculated the average quantity of immunoglobulin for each dilution and plotted these data graphically (see Appendix C, Figure 6.6) with plasma concentration on the X axis and absorbance on the Y. This graph identified a plasma dilution of 1/4000 (0.25 µL plasma in 1000 µL dilution solution; Figure 6.6) as the dilution with the steepest slope and thus was the dilution to analyze the remaining samples.

I performed the IgY assay using the same methods as for the pilot study described above, except instead of performing a serial dilution, I filled two wells with 100 µL of 1/4000 diluted plasma for each song sparrow as well as two wells per plate with 100 µL

pure dilution solution as a control. I took the average absorbance of the two wells for each sample to indicate the IgY concentration of the plasma sample.

2.3 Blood smear analysis

In July 2013, smears were Harleco ® stained (Hemacolor Stain Set; Millipore, 65044/93) following manufacturer's instructions, then rinsed with distilled water and allowed to air-dry. I viewed each blood smear under a light microscope using an 100x oil-immersion objective to assess leukocyte proportions and blood-borne parasite load.

Leukocyte proportions

I categorized the first 100 leukocytes as heterophils, eosinophils, basophils, lymphocytes or monocytes, with reference to Campbell and Ellis (2007) and recorded the number of thrombocytes observed, to calculate the number of thrombocytes per 100 leukocytes (Buehler et al., 2008).

Concentrations of different types of leukocytes indicate various aspects of immune function (Campbell, 1995). Heterophils are phagocytes, similar to mammalian neutrophils, which respond to novel microbial pathogens during the innate immune response (Juul-Madsen et al., 2008). Eosinophils, also associated with innate immunity, are cytotoxic cells capable of destroying other cells and large extracellular parasites (Juul-Madsen et al., 2008). Lymphocytes, typically the most common type of leukocyte, orchestrate antibody and cell-mediated functions of acquired immunity (Juul-Madsen et al., 2008). Monocytes are large leukocytes involved in innate immunity which develop into macrophages and provide nonspecific defence against pathogens by phagocytosing

pathogen-infected cells. They also link innate to acquired immunity by activating B and T lymphocytes (Demas et al., 2011). Thrombocytes are involved in blood clotting and in phagocytosis (Campbell, 1995).

Blood-borne parasite load

To assess blood-borne parasite load, I examined 10,000 erythrocytes on each slide under a 100x oil-immersion objective noting the number and presence of blood-borne parasites from genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Trypanosoma*, as well as microfilarian nematodes (family *Onchocerdciae*) using descriptions from Valkiunas (2004). Because *Plasmodium* and *Haemoproteus* cannot reliably be visually distinguished (Valkiunas, 2004) I counted these parasites collectively. For each individual, I categorized its overall infection status (infected by any blood-borne parasite or no detectable infection) and, for infected birds, noted the total parasite load of all parasites.

2.4 Stable hydrogen isotope analysis

Stable hydrogen isotopes from cleaned nail tissue were used as an indicator of migration distance. Nail samples were swirled around in 1.6 mL of 2:1 chloroform-methanol solution to remove any dirt or oils that might contaminate isotope analyses using. Excess chloroform-methanol was removed using a pipette and vials were left uncapped overnight in the fume hood to allow the nails to dry. Once dry, I weighed nails to 350 µg ± 10 µg (Mettler Toldeo MX5 Microbalance PSU30A-3). In cases where the nail was too large, I shaved off small amounts from the proximal end using a sterilized X-

acto knife blade. Nail samples weighing less than 350 mg [N= 1 (2013), N= 2 (2012)] were not analyzed. Once at the appropriate weight I crushed the nails in silver capsules and stored them in a sterile 96-well microplate at room temperature.

Samples were analyzed by Dr. Keith Hobson at the Stable Isotope Laboratory of Environment Canada, Saskatoon, Canada for nonexchangeable hydrogen using online continuous-flow isotope mass spectrometry (CF-IRMS) performed on a Micromass Isoprime mass spectrometer (Micro-mass UK, Manchester, UK) interfaced with a Eurovector elemental analyzer. The influence of exchangeable hydrogen was corrected by performing stable-hydrogen isotope analyses using the comparative equilibrium method whereby three keratin standards (Caribou Hoof Standard [CBS]: -197 ‰, Commercial Keratin [SPK]: -121.6 ‰ and Kudu Horn Standard [KHS]: -54.1 ‰) were used to correct for the effects of any H exchange with ambient water vapor (Wassenaar and Hobson, 2003). H isotopic measurements were performed on H_2 gas derived from high-temperature (1350 °C) flash pyrolysis of claw samples and keratin standards using continuous-flow isotope-ratio mass spectrometry. All keratin δD results are reported in units of per mil (‰) and normalized on the Vienna Standard Mean Ocean Water – Standard Light Antarctic Precipitation (VSMOW-SLAP) standard scale. Based on within-run replicate analyses of five of each keratin standard, the analytical precision is estimated to be ±2 ‰.

2.5 Statistical analysis

Data were analyzed in IBM SPSS version 21. Before beginning the statistical analysis, I explored the δD data to identify potential outliers as any individuals that

appeared to have made an unusually long or short migration based on stable isotope signatures. One female had an extremely low δD value of -116.77 ‰ as compared to the population average of -62.81 ± 1.97 ‰), suggesting that she had overwintered at the breeding grounds rather than migrating to the wintering grounds. Because this female was a statistical outlier (95th percentile of the δD distribution), and because δD values were non-normally distributed when this female was included (Shapiro-Wilk test of normality: $W_{54} = 0.949$, p= 0.023) but normally distributed when she was excluded ($W_{53} = 0.988$, p= 0.872), I excluded this female from further analyses. However, for analyzing patterns in migration (section 3.1), I report results both including and excluding this female. A second, male, subject had an unusually high δD value (-58.37‰), suggesting a much longer than average migration distance for his age. I excluded this individual from further analyses due to his extreme age (7 years, in contrast to the typical lifespan of 3-4 years at the study site).

I analyzed all variables for normality using the Shapiro-Wilk test. Wing length, phagocytosis scores, and agglutination scores were normally distributed. Mass, IgY concentrations, and parasite load (parasites per 10,000 erythrocytes) of parasitized birds required $\log_{10}$ transformations to yield normal distributions. Proportion data from leukocyte counts were not normally distributed and were not transformed because these data were entered into principal components analysis (PCA; described below), which does not require normally distributed variables (Quinn and Keough, 2002).

Before investigating sex and age effects on migratory distance, I checked for possible morphological covariates using Pearson's correlations. I found no evidence that morphology was related to migration distance (tarsus: Pearson's r= -0.067, n= 53, p=

0.63; wing: Pearson's r= -0.235, n= 53, p= 0.09; mass: Pearson's r= 0.195, n=53, p= 0.16). The weak relationship between migration distance and wing chord was a result of the differences in wing length between sexes where males have a greater average wing length than females (t_{47}=8.00, p<0.0001; mean±sem wing chord: males, 65.85±0.387 cm [n=28]; females, 62.12 ±0.262 cm [n=28]) so any relationship between wing length and migration distance likely reflects sex differences in migration distance. Because morphology was unrelated to migration distance, I used no morphological covariates when investigating relationships between migration distance and sex and age.

For the subset of birds sampled in both 2012 and 2013, I assessed migratory consistency using a Pearson correlation on δD values obtained in 2012 versus 2013. I also conducted a paired t-test on δD values obtained in 2012 versus 2013, to further examine differences in migratory tendency between years. Because these tests indicated no significant difference in migration distance between years (Levene's test: test: $F_{1,97}$=6.09, p=0.015; independent samples t-test assuming unequal variances: t_{73}=0.004, p=0.99; mean±sem δD: 2012, -61.78 ±2.97, n=46; 2013, -61.79 ±1.71, n=53), years were pooled when testing for sex and age differences using a linear mixed model analysis with age and sex as continuous and categorical predictors, respectively, migratory distance (δD) as the dependent variable, and bird ID as a random factor to control for repeated samples from some (n=13) individuals. Least significant variables were removed from models until only significant variables remained. In the case where post-hoc examinations were necessary to investigate significant interaction terms, datasets were split by sex, infection status, or age (continuous in the original model, split by major age classes for post-hoc examination). Variables included in post-hoc examinations are those which remained in

the final original model. Least significant variables were once again removed until only significant variables remained in the model. This was completed for all subsequent models.

To examine how migration distance influences the incidence of infection with a blood-borne parasite, I performed a generalized linear model regression with a binomial distribution. For this model, δD, sex, and age were independent variables, infection status (parasitized or not) was the binary dependent variable, and date and age as a factor and covariate, respectively. To further examine whether blood-borne parasite load might be influenced by migration distance, birds were first categorized by infection status (infected with any parasite or no detectable infection). I examined only the subset of birds that were found to be parasitized. I used a linear mixed model regression with δD as the independent variable, parasite load as the dependent variable, capture date and age as covariates and sex as a factor.

To explore the relationships between the different types of leukocytes (lymphocytes, heterophils, eosinophils, monocytes) and thrombocytes, I generated a Spearman's correlation matrix. Cell types found to significantly correlate with one or more other cell type (all) were entered into a principal components analysis (PCA) using a correlation matrix and Varimax rotation to maximize the variance of the loadings. Principal components with eigenvalues greater than one were retained for subsequent mixed model analyses. These models used migration distance and capture date as independent variables and the leukocyte PCs as dependent variables. No leukocytes were included in independent models.

To test the hypothesis that migratory distance is related to immune allocation, I conducted a series of linear mixed model analyses on the 2013 dataset with migratory distance (δD) as the dependent variable, an immune measure (phagocytic activity, agglutination score, IgY concentration, PC1, and PC2) as the dependent variable, and sex and age as a factor and a covariate, respectively. For models of immune variables found to be significantly associated with capture date (phagocytic activity, IgY, PC2) or time of day (agglutination), I also included these variables as covariates. Additional morphological covariates were included when they were significantly correlated with the dependent immune variable being modelled: body mass as a covariate for the phagocytosis and PC2 models.

To examine how capture date (phases of the annual cycle) influences the incidence of infection with a blood-borne parasite, I performed a generalized linear model with a binomial distribution. For this model, capture date was the independent variable, infection status (parasitized or not) was the binary dependent variable, and sex, δD and age as a factor and covariates, respectively. When examining seasonal variation in parasite load, the *a priori* hypothesis that parasite load will increase with capture date (based on empirical findings in other species: Møller et al., 2004; Rätti et al., 1993) warranted using a one-tailed test (alpha = 0.1). I used a linear mixed model regression with capture date as the independent variable, parasite load as the dependent variable, δD and age as covariates and sex as a factor.

I examined seasonal variation in immune allocation using linear mixed models with an immune variable (phagocytic activity, agglutination score, IgY concentration, PC1, and PC2) as the dependent variable, date as the independent variable, and sex and

age as a factor and a covariate, respectively. Additional covariates were included when they were significantly correlated with the dependent variable being modelled: body mass for phagocytosis and leukocyte PC2 models.

3   *Results*

3.1 Intraspecific variation in migration distance

Year-to-year variation in migration distance

For the 13 birds sampled in both 2012 and 2013, migration distance in one year was not correlated to migration distance the next year (δD: Pearson's r=0.34, n=13, p=0.26; Figure 3.1). An independent samples t-test (assuming unequal variances; Levene's test: $F_{1,88}$=5.78, p=0.018) also indicated no significant difference in migration distance between years when including all birds, excluding birds in 2013 which had nail tissue also sampled in 2012 (t_{80}=0.11, p=0.91; mean±sem δD: 2012, -61.71 ±2.90, n=47; 2013, -62.09 ±1.96, n=43). Because the population migrated similar distances on average each year, years were pooled when testing for sex and age differences, and bird ID was included as a random factor to control for repeated samples from some (n=13) individuals.

Effects of Sex and Age

When data from 2012 and 2013 were pooled, there was no main effect of sex nor year but there was a significant main effect of age where migration distance decreased with age, and a significant interaction between sex and age whether the outlier females was included (Linear mixed model, sex: $F_{1,89}$=-0.77, p=0.38; age: $F_{1,89}$=5.21, p=0.025; sex*age: $F_{1,89}$=3.99, p=0.049) or not (Linear mixed model, sex: $F_{1,88}$=0.71, p=0.40; age: t_{88}=-0.05, p=0.96; sex*age: $F_{1,88}$=5.07, p=0.027; Figure 3.2). To explore this interaction, I examined the effect of age for each sex separately. Female migration distance did not
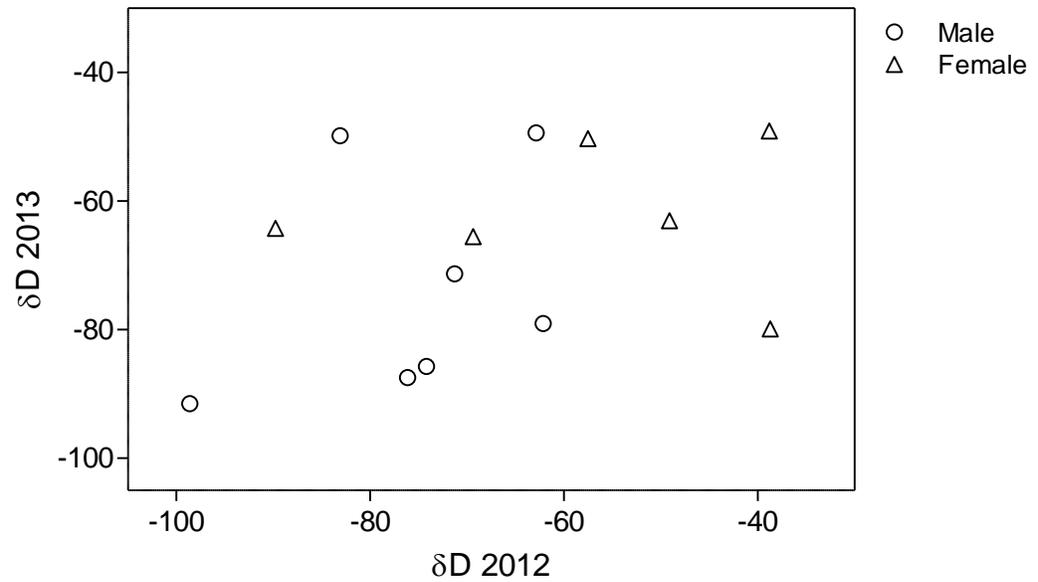
Figure 3.1 Migration distances (inferred from δD) of song sparrows (n=13) captured near Newboro, ON, for the winter of 2011-2012 (δD 2012) plotted against the migration distances of the same individuals for the winter of 2012-2013 (δD 2013).
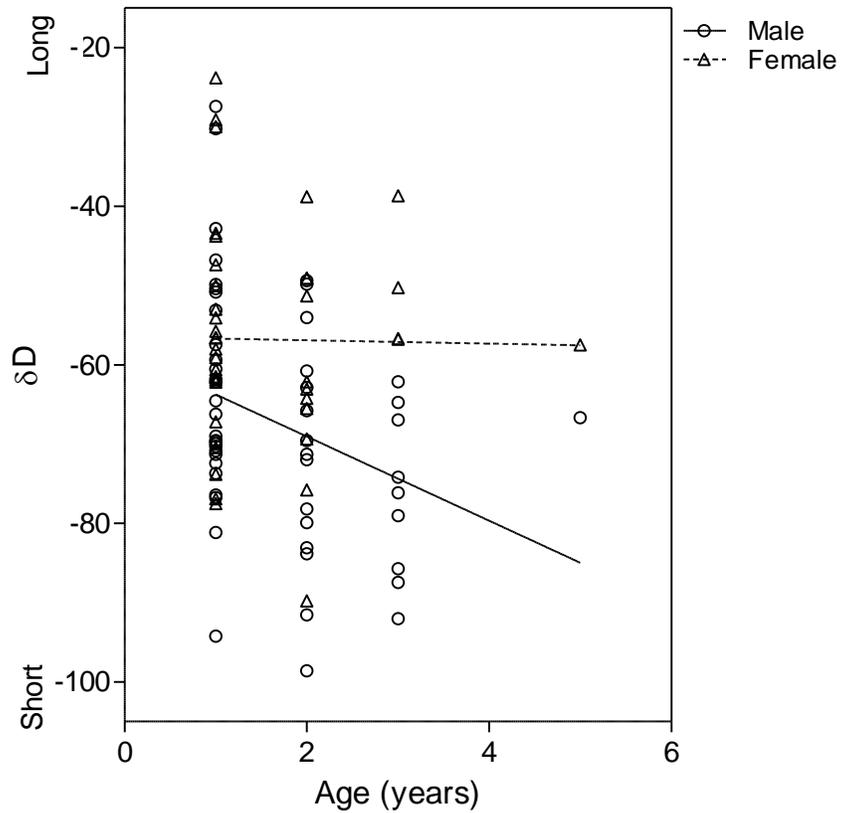
Figure 3.2 Migration distance (as inferred from δD) as a function of age (years) for male (n=58) and female (n=42) song sparrows caught near Newboro, ON in 2012 and 2013 (n=87 individuals, including 13 birds sampled in both years). Age and sex interacted to predict migration distance: migration distance did not vary with age for females, but migration distance decreased with age for males. Lines represent lines of best fit.

vary with age but male migration distance decreased with age (Linear mixed model, female: age: $F_{1,38}=0.003$, p=0.95; male: age: $F_{1,50}=7.58$, p=0.008).

3.2 Leukocyte principal component analysis (PCA)

Leukocyte correlations

Several leukocyte counts were significantly correlated with each other. The proportion of lymphocytes was negatively correlated with both heterophil and eosinophil proportions, while the proportion of monocytes was positively correlated with the number of thrombocytes per 100 leukocytes (Table 3.1).

PCA loadings

Due to the intercorreations among leukocyte types (Table 3.1) leukocyte counts were entered into a principal component analysis (PCA) to reduce dimensionality. The PCA identified two factors with eigenvalues greater than one that cumulatively accounted for 65.3% of the overall variance in leukocyte counts (Table 3.2). High positive values of PC1 were associated with a low number of lymphocytes (associated with acquired immunity) and a high number of heterophils and eosinophils (associated with innate immunity; Figure 3.3). High positive values of PC2 were associated with a high proportion of monocytes and a high number of thrombocytes (Figure 3.3), both of which function in innate immunity, but also with additional functions in the immune response.

Table 3.1 Spearman's correlation matrix for the first 100 leukocytes counted on blood smears of song sparrows caught near Newboro, ON in April – May 2013. Values underlined and in bold denote statistically significant correlations at (α= 0.05). N = 54 birds.

	Lymphocyte	Heterophil	Eosinophil	Monocyte	Thrombocyte
Lymphocyte		**-0.676**	**-0.502**	-0.260	0.181
Heterophil			0.171	-0.054	-0.203
Eosinophil				0.156	0.029
Monocyte					**0.327**
Thrombocyte					

Table 3.2 Factor loadings for principal components analysis (PCA) of leukocyte proportions for 54 song sparrows caught near Newboro, ON, in April – May 2013. PC1 was associated positively with proportions of heterophils and eosinophils and negatively with proportion of lymphocytes. PC2 was associated positively with proportion of monocytes and number of thrombocytes. Other principal components (PC3, 4, 5) each explained less than 20% of the variance in leukocyte counts, had eigenvalues less than one and were not retained for further analysis.

Response	PC1	PC2
Lymphocyte	-0.973	-0.012
Heterophil	0.728	-0.348
Eosinophil	0.580	0.277
Monocyte	0.173	0.773
Thrombocyte	-0.161	0.775
Totals		
Variance per component (%)	37.58	27.72
Cumulative variance (%)	37.58	65.30
Eigenvalue	1.88	1.39
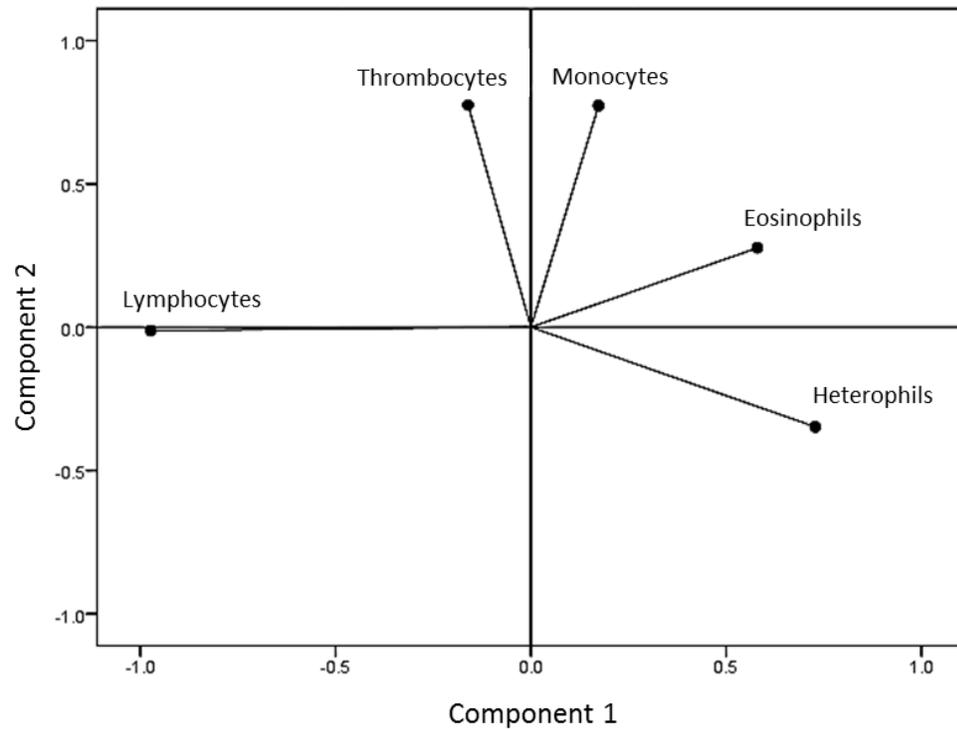
Figure 3.3 Component plot in rotated space of the leukocyte principal component analysis (PCA). High positive values of component 1 (PC1) indicate high proportions of heterophils and eosinophils and low proportion of lymphocytes (interpreted as high innate immunity and low adaptive immunity). High positive values of component 2 (PC2) indicate a high proportion of monocytes and a high number of thrombocytes.

3.3 Effects of migration distance

Migration distance and parasitism

Migration distance was positively related to infection status, but not parasite load in the subset of birds found to be parasitized. Migration distance was related to infection status as a main effect and also related to infection status in an interaction with age (coded as a continuous variable; Table 3.3). In the interaction of migration distance and age that was related to infection status, the incidence of infection increased with migration distance for after second year (ASY) birds but not for second year (SY) birds (age split into major age classes; Post-hoc Binomial logit GLM, SY: whole model: χ^2=6.04, n=34, p=0.014; sex: χ^2=5.45, n=34, p=0.020; ASY: whole model: χ^2=6.29, n=16, p=0.012; δD: χ^2=3.98, n=16, p=0.046; Figure 3.4). Migration distance was also related to infection status in an interaction with capture date (Table 3.3); however, date and migration distance were not correlated for infected or uninfected birds (Linear mixed model, infected birds: age: $F_{1,23}$=5.64, p=0.026; uninfected birds: no significant variables left in the model; Figure 3.5). Of the birds that were parasitized, parasite load was not related to migration distance (Table 3.4).

Migration distance and immunity

I found mixed and sex-specific evidence for measures of innate immunity being related to migration distance. Migration distance was not related to agglutination (Table 3.5). However, migration distance was related to phagocytic activity of macrophages in an interaction with sex (Table 3.6). This interaction was due to female phagocytic activity

Table 3.3 Binomial logit generalized linear model of infection status as a function of sex, age, migration distance (inferred from δD), and date for song sparrows caught near Newboro, ON, in April – May 2013. The most parsimonious model is reported. Values underlined and in bold denote statistically significant correlations at (α= 0.05).

Dependent Variable	Independent Variable	χ^2	N	p
Infection status	sex	0.065	52	0.800
	age	0.617	52	**0.013**
	δD	4.970	52	**0.026**
	date	4.970	52	**0.026**
	sex x date	4.570	52	**0.033**
	age x δD	5.230	52	**0.022**
	δD x date	4.070	52	**0.044**
Eliminated variables:	sex x δD, sex x age, age x date.			
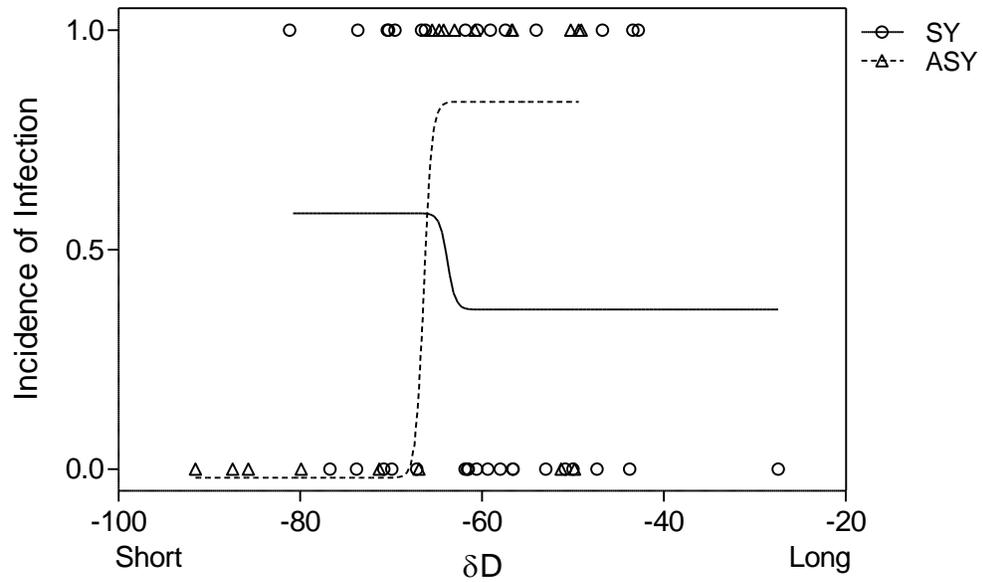
Figure 3.4 Incidence of infection with a blood-borne parasite (per 10 000 red blood cells) as a function of migration distance (inferred from δD) in second-year (SY; n=34) and after-second-year (ASY; n=21) song sparrows caught near Newboro, ON, in April – May 2013. Age and migration distance interacted to predict infection status: the incidence of infection increased with migration distance for ASY birds but not for SY birds. Lines represent logistic regression curves.
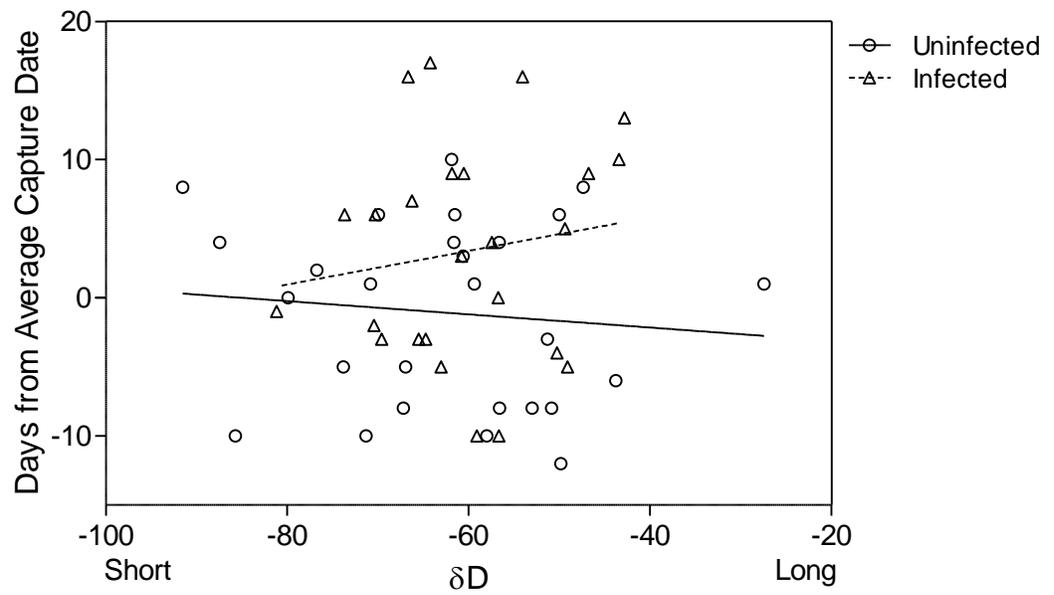
Figure 3.5 Capture date (recorded as the number of days from the average capture date) as a function of migration distance (inferred from δD) for uninfected (n=29) and infected (n=26) song sparrows caught near Newboro, ON, in April – May 2013. Migration distance and capture date interacted to predict infection with blood-borne parasites. Lines represent lines of best fit.

Table 3.4 Linear mixed model of parasite load (per 10,000 red blood cells) as a function of sex, age, migration distance (inferred from δD), and capture date for song sparrows caught near Newboro, ON, in April – May 2013. No significant variables remained in the model; statistics reported are those of main effects prior to elimination from the model.

Dependent Variable	Independent Variable	*F*	d.f.	*p*
Parasite load	sex	0.230	1, 21	0.630
	age	1.010	1, 22	0.330
	δD	0.049	1, 20	0.830
	date	3.000	1, 23	0.096
Eliminated variables:	sex x age, sex x δD, sex x date, age x δD, age x date, δD x date.			

Table 3.5 Linear mixed model of agglutination score as a function of sex, age, migration distance (inferred from δD), and time of day for song sparrows caught near Newboro, ON, in April – May 2013. No significant variables remained in the model; statistics reported are those of main effects prior to elimination from the model.

Dependent Variable	Independent Variable	*F*	d.f.	*p*
Parasite load	sex	0.390	1, 46	0.530
	age	0.880	1, 51	0.350
	δD	0.430	1, 47	0.510
	time	3.060	1, 52	0.086
Eliminated variables:	sex x age, sex x δD, , sex x time, age x δD, age x time, δD x date.			

Table 3.6 Linear mixed model of phagocytic activity as a function of sex, age, migration distance (inferred from δD), capture date, and mass for song sparrows caught near Newboro, ON, in April – May 2013. The most parsimonious model is reported. Values underlined and in bold denote statistically significant correlations at (α= 0.05).

Dependent Variable	Independent Variable	*F*	d.f.	*p*
Proportion Phagocytosis	sex	5.030	1, 44	**0.030**
	age	5.470	1, 44	**0.024**
	δD	0.680	1, 44	0.410
	date	23.41	1, 44	**<0.001**
	mass	1.230	1, 44	0.270
	sex x δD	5.030	1, 44	**0.030**
	age x date	6.720	1, 44	**0.013**
	age x mass	5.310	1, 44	**0.026**
Eliminated variables:	sex x mass, date x mass, δD x date, δD x age, sex x age, sex x date, δD x mass.			

being unaffected by migration distance but male phagocytic activity decreased with increasing migration distance (Table 3.7; Figure 3.6). IgY concentration, a measure of acquired immunity, was not related to migration distance (Table 3.8).

I found no evidence that migration distance affected leukocyte proportions. Neither leukocyte PC1 nor leukocyte PC2 was significantly related to migration distance (Table 3.9).

3.4 Seasonal variation in parasites and immunity

Seasonal variation in parasitism

Both the incidence of a bird being infected with a blood-borne parasite and an individual's total parasite load (infected individuals only), increased with day of capture (the number of days from the overall average capture date). Birds caught later in the breeding season were more likely to be infected and this effect interacted with sex (Table 3.3). Specifically, the incidence of infection increased with capture date for males but not for females (Post-hoc binomial logit GLM, males: whole model: χ^2=9.79, n=28, p=0.014; date: χ^2=6.07, n=28, p=0.014; females: whole model: χ^2=5.72, n=27, p=0.017; age: χ^2=3.82, n=27, p=0.050; Figure 3.7). Finally, among the subset of infected individuals, capture date was positively related to parasite load (Linear mixed model, date: $F_{1,23}$=3.00, p=0.048; Figure 3.8).

Table 3.7 Post-hoc linear mixed models of phagocytic activity as a function of age, migration distance (inferred from δD), capture date, and mass modelled separately for male and female song sparrows caught near Newboro, ON, in April – May 2013. The most parsimonious models are reported. Values underlined and in bold denote statistically significant correlations at (α= 0.05).

Dependent Variable	Independent Variable	*F*	d.f.	*p*
Males:				
Proportion Phagocytosis	date	13.66	1, 24	**0.001**
	δD	5.320	1, 24	**0.030**
Eliminated variables:	age, mass, age x date, age x mass			
Females:				
Proportion Phagocytosis	age	4.580	1, 22	**0.044**
	date	22.64	1, 22	**<0.001**
	mass	1.830	1, 22	0.190
	age x mass	4.560	1, 22	**0.044**
Eliminated variables:	δD, age x date			
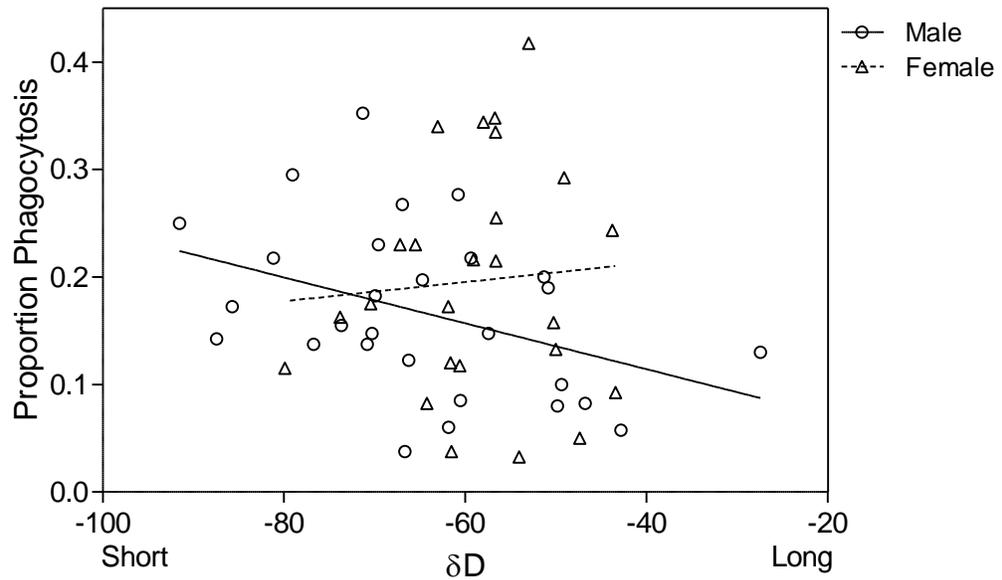
Figure 3.6 Macrophage phagocytic activity as a function of migration distance (inferred from δD) for male (n=28) and female (n=27) song sparrows caught near Newboro, ON, in April – May 2013. Sex and migration distance interacted to predict macrophage phagocytic activity: phagocytic activity was unaffected by migration distance for females, but phagocytic activity decreased with migration distance for males. Lines represent lines of best fit.

Table 3.8 Linear mixed model of the circulating concentration of immunoglobulin Y (IgY) as function of sex, age, migration distance (inferred from δD), and capture date for song sparrows caught near Newboro, ON, in April – May 2013. No significant variables remained in the model; statistics reported are those of main effects prior to elimination from the model.

Dependent Variable	Independent Variable	*F*	d.f.	*p*
IgY	sex	0.700	1, 46	0.410
	age	0.100	1, 51	0.750
	δD	0.990	1, 47	0.540
	date	2.790	1, 52	0.100
Eliminated variables:	sex x age, sex x δD, sex x date, age x δD, age x date, δD x date.			

Table 3.9 Linear mixed model of leukocyte PC1 (the proportion of heterophils and eosinophils against lymphocytes) and PC2 (the proportion of monocytes and number of thrombocytes) as a function of sex, age, and δD for PC1 and sex, age, migration distance (inferred from δD), capture date, and mass for PC2. For PC1, no significant variables remained in the model; statistics reported are those of main effects before elimination from the model. For PC1, the most parsimonious model is reported. Data is from song sparrows caught near Newboro, ON, in April – May 2013 Values underlined and in bold denote statistically significant correlations at (α= 0.05).

Dependent Variable	Independent Variable	F	d.f.	p
PC1	sex	<0.001	1, 47	0.990
	age	0.270	1, 49	0.600
	δD	0.770	1, 50	0.380
Eliminated variables:	sex x age, sex x δD, age x δD.			
PC2	sex	8.41	1, 48	**0.006**
	mass	9.31	1, 48	**0.004**
	date	5.13	1, 48	**0.028**
	sex x date	9.10	1, 48	**0.004**
	mass x date	4.67	1, 48	**0.036**
Eliminated variables:	δD, age, δD x mass, δD x sex, δD x date, δD x age, sex x age, age x date, age x mass, sex x mass.			
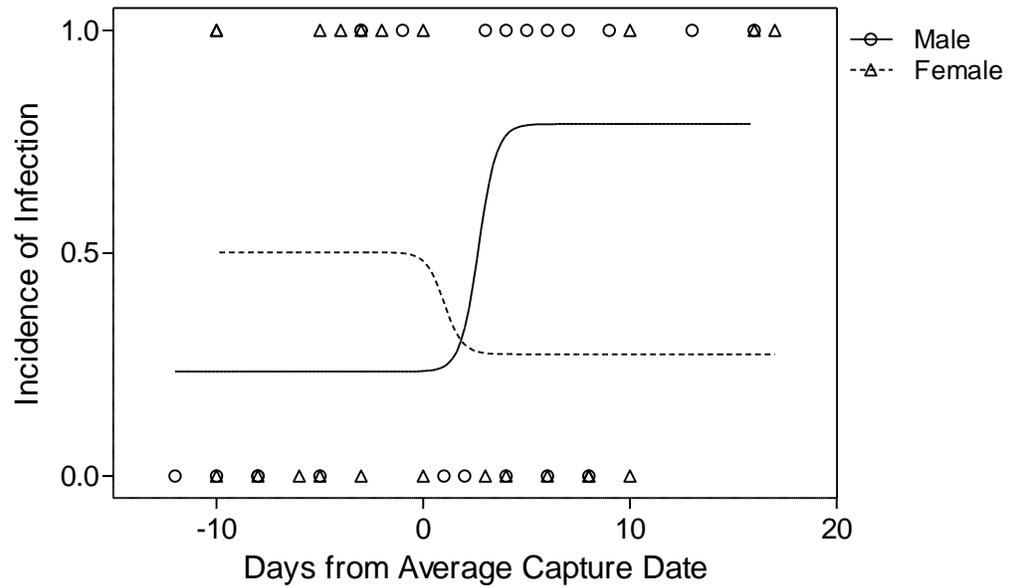
Figure 3.7 Incidence of infection with a blood-borne parasite as a function of capture date (recorded as the number of days from the average day of capture) for male (n=28) and female (n=27) song sparrows caught near Newboro, ON, in April – May 2013. Sex and capture date interacted significantly to predict infection status: the incidence of infection increased significantly with capture date for males but not for females. Lines represent logistic regression curves.
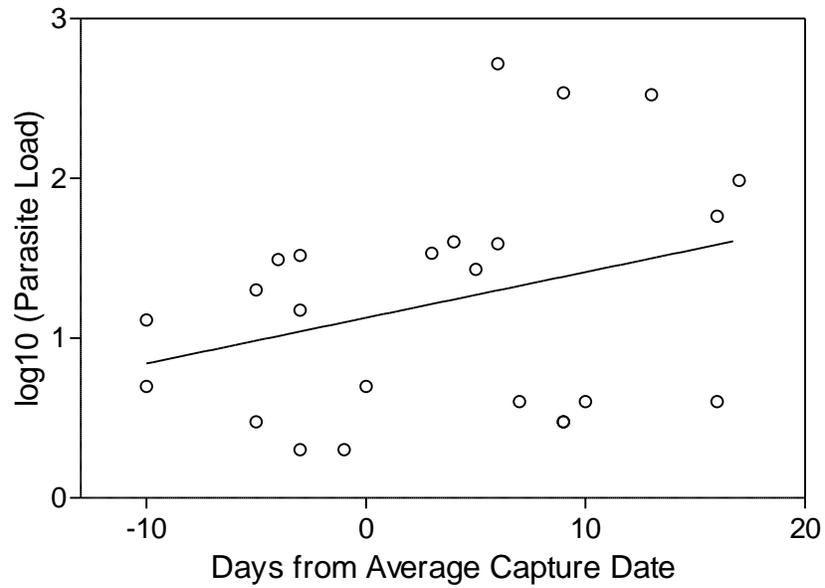
Figure 3.8 Total blood-borne parasite load (per 10,000 red blood cells) as a function of date (recorded as the number of days from the average capture date) of infected song sparrows (n=25) caught near Newboro, ON, in April – May 2013. Birds captured later in the season had higher total parasite loads. Line represents a line of best fit.

Seasonal variation in immunity

One measure of innate immunity decreased throughout the field season (i.e. with increasing capture date) while acquired immunity did not. Agglutination score (a measure of innate immunity) was not related to capture date (Table 3.10). However, macrophage phagocytosis (another aspect of innate immunity) decreased significantly with capture date, and capture date was also related to macrophage phagocytosis in an interaction with age (coded as a continuous variable) (Table 3.11). To further examine the interaction of capture date and age, I categorized birds into two discrete age classes (i.e. SY and ASY). Macrophage phagocytosis decreased with date for both age classes, but more strongly for SY than ASY birds (Post-hoc linear mixed model, SY: date: $F_{1,32}=62.18$, p<0.001; ASY: date: $F_{1,17}=4.47$, p=0.050; mass: $F_{1,17}=5.21$, p=0.036; Figure 3.9). A linear mixed model indicated IgY concentration (acquired immunity) was not related to capture date (Figure 3.10) but capture date explained the most variation in IgY concentrations (Table 3.12).

PC1 (indicating the proportion of heterophils and eosinophils relative to lymphocytes) was not related to date of capture (Table 3.13). However, capture date was negatively related to PC2 (indicating the proportion of monocytes and number of thrombocytes) and capture date was also related to PC2 in an interaction with sex (Table 3.9). In the interaction of date with sex, PC2 decreased with capture date for males but not females (Post-hoc linear mixed model, males: date: $F_{1,25}=18.31$, p<0.001; females: mass: $F_{1,24}=14.97$, p=0.001; Figure 3.11).

Table 3.10 Linear mixed model of agglutination score as a function of sex, age, and capture date for song sparrows caught near Newboro, ON, in April – May 2013. No significant variables remained in the model; statistics reported are those of main effects prior to elimination from the model.

Dependent Variable	Independent Variable	*F*	d.f.	*p*
Parasite load	sex	0.063	1, 49	0.800
	age	1.140	1, 51	0.290
	date	2.28	1, 52	0.140
Eliminated variables:	sex x age, sex x date, age x date.			

Table 3.11 Linear mixed model of phagocytic activity of macrophages as a function of sex, age, migration distance (inferred from δD), capture date, and mass for song sparrows caught near Newboro, ON, in April – May 2013. The most parsimonious model is reported. Values underlined and in bold denote statistically significant correlations at (α= 0.05).

Dependent Variable	Independent Variable	*F*	d.f.	*p*
Proportion Phagocytosis	age	5.61	1, 49	**0.022**
	date	5.48	1, 49	<0.001
	mass	1.87	1, 49	0.180
	age x date	3.93	1, 49	**0.050**
	age x mass	5.48	1, 49	**0.023**
Eliminated variables:	sex, sex x mass, sex x age, sex x date, mass x date.			
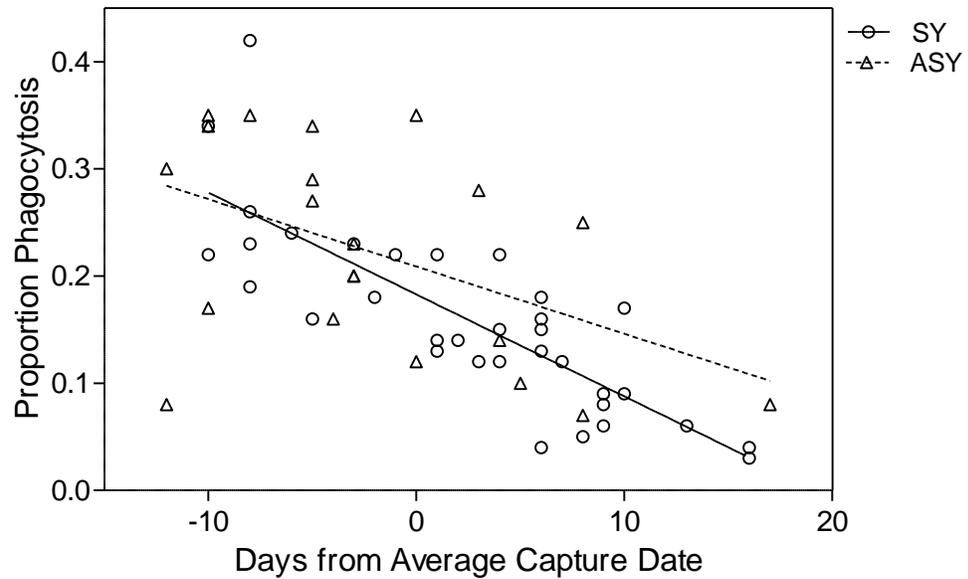
Figure 3.9 Macrophage phagocytic activity as a function of date (recorded as the number of days from average capture date) for second-year (SY; n=34) and after-second-year (ASY; n=21) song sparrows caught near Newboro, ON, in April – May 2013. Age and capture date interacted to predict phagocytic activity of macrophages: phagocytic activity decreased with capture date for both age classes, but more strongly for SY than ASY birds. Lines represent lines of best fit.
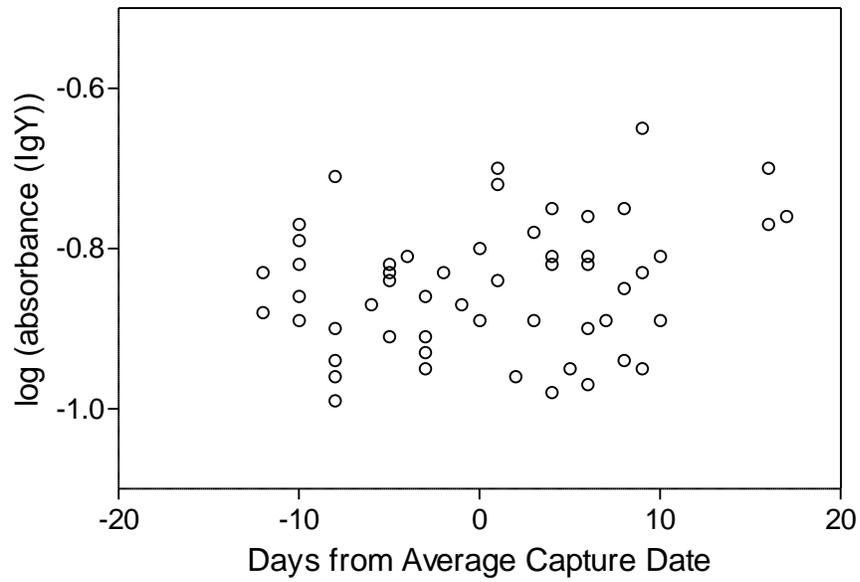
Figure 3.10 log absorbance units [indicating the circulating concentration of immunoglobulin Y (IgY)] as a function of date (recorded as the number of days from average capture date) in male (n=28) and female (n=27) song sparrows caught near Newboro, ON, in April – May 2013. IgY tended to increase with capture date, although there is no significant relationship.

Table 3.12 Linear mixed model of the circulating concentration of immunoglobulin Y (IgY) as a function of sex, age, and capture date for song sparrows caught near Newboro, ON, in April – May 2013. No significant variables remained in the model; statistics reported are those of main effects prior to elimination from the model.

Dependent Variable	Independent Variable	*F*	d.f.	*p*
IgY	sex	0.400	1, 49	0..530
	age	0.100	1, 51	0.750
	date	2.790	1, 52	0.100
Eliminated variables:	sex x age, sex x date, age x date.			

Table 3.13 Linear mixed model of PC1(the proportion of heterophils and eosinophils against lymphocytes) as a function of sex, age, and capture date for song sparrows caught near Newboro, ON, in April – May 2013. No significant variables remained in the model; statistics reported are those of main effects prior to elimination from the model.

Dependent Variable	Independent Variable	*F*	d.f.	*p*
PC1	sex	0.010	1, 51	0..960
	age	0.640	1, 49	0.430
	date	0.410	1, 52	0.520
Eliminated variables:	sex x age, sex x date, age x date.			
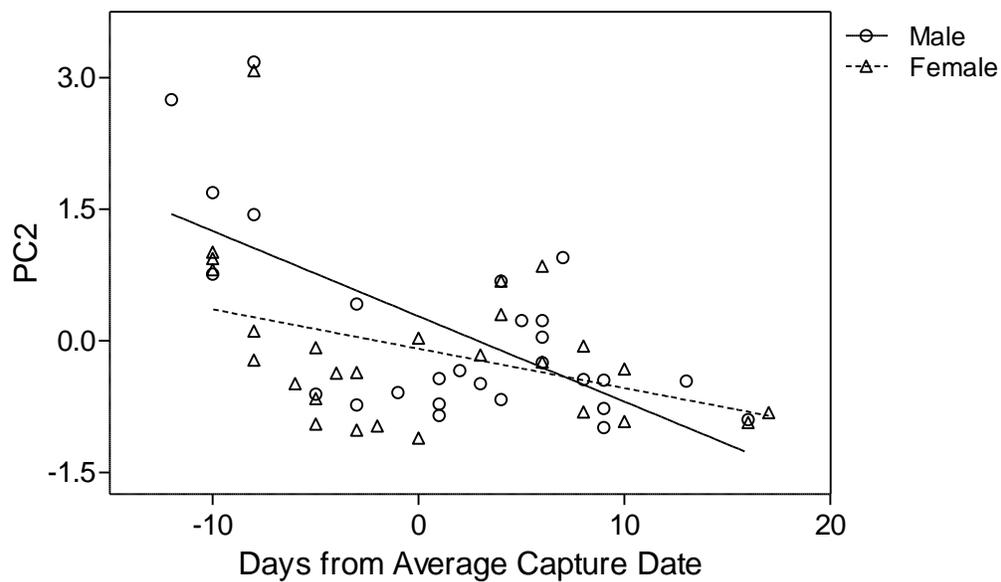
Figure 3.11 Leukocyte PC2 as a function of date (recorded as the number of days from the average capture date) in male (n=28) and female (n=27) song sparrows caught near Newboro, ON, in April – May 2013. High values of PC2 reflect high abundance of monocytes and thrombocytes. Capture date interacted significantly with sex in predicting leukocyte PC2: PC2 decreased with capture date for both sexes, but more strongly in males than in females. Lines represent lines of best fit.

4 *Discussion*

Overview

Migration distance was not correlated between years for the subset of birds sampled in both 2012 and 2013 (Figure 3.1). Stable hydrogen isotope ratios indicated substantial variation in migration distance associated with age, where young-of-the-year migrated farther than adults, but no significant main effect of sex (Figure 3.2). I found some support for the hypothesis that long-distance migrants are at greater risk of parasitism, as the incidence of infection with a blood-borne parasite increased with migration distance (Figure 3.4). One aspect of innate immunity (macrophage phagocytosis) decreased as migration distance increased (Figure 3.6). However, this relationship was observed only for males and not for other measures of innate or acquired immunity. This pattern opposes my original hypothesis that investment in innate immunity should increase with migration distance. Instead, this finding supports the hypothesis that the energetic costs of migration may constrain the ability to invest in some aspects of immunity.

There was also variation in the incidence of being parasitized, total parasite load, and the balance between innate and acquired immune function in relation to the phases of the annual cycle. Specifically, the incidence of infection by blood-borne parasites (for males) and the total blood-borne parasite load (of parasitized birds for both sexes) increased as birds recovered from migration and transitioned into the breeding phase of the annual cycle (Figure 3.7, 3.8). Innate immune function decreased throughout the transition (Figure 3.11) while acquired immunity tended to increase (although not significantly so; Figure 3.10) suggesting variation in immune function may be related to

the familiarity of parasites a bird encounters. Furthermore these results indicate immunity may be important during migration as innate immune function was greatest when birds were recovering from migration. Both migration distance and the phases of the annual cycle were implicated in incidence of parasitism and in some parameters of immunity, suggesting that immune function may vary in response to changes in the risk of encountering new or familiar parasites.

4.1 Intraspecific variation in migration distance

δD values obtained from song sparrow claw sampled varied from -98.61 to -23.81 ‰ in 2012, and from -92.02 to -27.43‰ in 2013. Comparing these signatures to those of December precipitation for North America (Meehan et al., 2004; Figure 1.2) suggests that the study birds wintered at latitudes between Pennsylvania and Louisiana in 2012, and between Maryland and Louisiana in 2013 which is consistent with the recapture of some of our banded birds during previous winters in Maryland and Tennessee. However, the wintering latitudes inferred from the isotope data should be interpreted with caution in regards to isotope fractionation, as described in introduction (evaluating avian migration distance).

With the exception of one outlier female, who appeared to overwinter at or near the summer study site during the 2012/2013 winter, all other birds appeared to migrate to lower latitudes suggesting this population exhibits a differential migration strategy, rather than partial migration. Young-of-the-year made the longest migrations, consistent with my original prediction. That is, birds sampled in their first breeding season (SY) had claw δD signatures characteristic of longer (more southerly) wintering locations than did older

birds (ASY; Figure 3.2). I observed no main effect of sex on migration distance. Instead, migration distance was related to sex in an interaction with age such that the oldest males made the shortest migrations (Figure 3.2), consistent with findings from other species [dark-eyed juncos (Ketterson and Nolan, 1985), blue tits (Smith and Nilsson, 1987), and house finches (Belthoff and Gauthreaux, 1991)].

Age and sex-specific variation in migration distance might reflect differences in body size, social dominance, or optimal arrival date, and each of these factors has been proposed as a cause of intraspecific variation in migration distance (Ketterson and Nolan, 1987). I found little support for the body size hypothesis to explain variation in migration distance in this population because this species is monomorphic except for males having longer wings than females (Chapter 2). However, because over-wintering male song sparrows are larger than females in some populations (Nice, 1937), I cannot fully exclude the possibility that larger body size may allow older males to winter at higher latitudes than females. Next, my findings are partially consistent with the social dominance hypothesis because male song sparrows are socially dominant over females (Knapton and Krebs, 1976; Arcese and Smith, 1985) and adults are dominant over younger birds (Arcese and Smith, 1985). Socially subordinate birds may be forced to migrate longer distances to avoid competition with dominant birds on the wintering grounds, and I found that older males (socially dominant; Nice, 1937) tended to migrate the shortest distances. Finally, my data provide little support for the arrival time hypothesis. While males establish and defend territories in this species (Nice, 1937; 1942) to benefit from obtaining more desirable territories (Cristol et al., 1999), I found no significant sex difference in migration distance nor was migration distance correlated with arrival date

(as inferred from capture date). Overall, the adaptive significance of the observed age-by-sex interaction in migration distance remains elusive, and further investigation is required before definite conclusions can be drawn.

Year-to-year variation in migration distance

While individuals do not appear to be consistent in their migration distance, the population maintains a similar average migration distance year to year. For the subset of song sparrows sampled in both spring 2012 and spring 2013, I observed no significant correlation between individuals' migration distances as assessed by δD in the in spring of 2012 and spring of 2013. The variation at the individual level may in part be due to males (but not females) making shorter migrations as they age (Figure 3.2); however, this sample size was limited (N =13) and a larger sample size may be required to conclusively assess repeatability of migration distance. The consistency in migration distance as a population, despite variation at the individual level, may be maintained by the long migrations of new young recruits balancing the shorter migrations made by males as they age.

In addition to the age- and sex-specific variation noted above, individual variation in migration distance year-to-year may reflect local weather conditions encountered during fall migration. Specifically, individuals might adjust their migration distance to avoid severe weather. While conditions during November 2012 were an average of 3 ˚C cooler than in November 2011 (National Oceanic and Atmospheric Administration, United States Department of Commerce), I observed no difference in the average migration distance in the two study years. Over a longer time-scale, changes in climate

are anticipated (Walther et al., 2002) and bird migratory behaviour appears to be changing with a shift toward earlier arrival for spring migrations (Crick, 2004) and shorter migration distances (Visser et al., 2009). Additionally, among species, long-distance migrants are advancing their autumn migration whereas short-distance migrants are delaying their autumn migration (Jenni and Kéry, 2003). Long-term information regarding migration distances and arrival times for this population are of interest to evaluate how individual variation in migration distance and arrival may be affected by the warming climate.

4.2 Effects of migration distance

Migration distance and parasitism

The incidence of being parasitized once captured on the breeding grounds increased with increasing migration distance, as predicted and as previously observed across waterfowl species (Figuerola and Green, 2000). However, my data are the first to report of this effect within a species. Adult song sparrows appear to be responsible for this relationship as the incidence of infection increases with migration distance for ASY birds but did not vary with migration distance for SY birds. Young purple martins (*Progne subis*) had a lower incidence of infection than adults on the breeding grounds and this relationship was explained by parasites causing high mortality in young birds which had not previously encountered a parasite while under the stress of their first migration (Davidar and Morton, 1993) since younger age classes are more likely to die from infections (Atkinson and Van Riper, 1991). This relationship may also explain why the incidence of parasitism did not vary with migration distance for SY birds in my

population; the incidence of infection may not have increased with migration distance for SY birds because these individuals likely died along their journey and were not present in my data. Such reasoning supports the idea that migration may lower infectious disease risk by removing infected individuals from the population (migratory culling) as proposed in monarch butterflies (*Danaus plexippus*; Bradley and Altizer, 2005). However, because only young birds (SY), and not adults (ASY), may demonstrate such a relationship my data indicate differential migratory culling. This high mortality in SY birds may act as an important source of selection, for either high tolerance of infection (Altizer et al., 2011) or enhanced immunity (Davidar and Morton, 1993). Furthermore, because the presumed death of parasitized SY birds occurred only in those migrating long distances, these results suggest migratory culling may be related to migration distance, at least for young birds, and long-distance transport of parasites may not be possible for young age classes. Previous research in western sandpipers (*Calidris mauri*) suggests that infected birds may be able to migrate while infected (Nebel et al., 2013) and my findings suggest only older age classes may be able to do so for long distances.

Unlike the incidence of infection, the total parasite loads of infected birds were not related to migration distance. This is likely explained by the relationship between the stage of infection and parasite load. Malarial parasites have acute and chronic stages of infection that differ with respect to the number of parasites circulating in the blood. In this population of song sparrows, the acute stage of *Plasmodium* infection peaks for 12 - 21 days after inoculation at approximately 30 infected cells per 10 000 red-blood cells but then drops to 4 per 10,000 in the chronic stage (Sarquis-Adamson and MacDougall-Shackleton, 2014, In Review). Consequently, the parasite load of a given bird may reflect

the stage of infection rather than severity, at least for malaria infections

(*Plasmodium/Haemoproteus*), which was the most common parasite found in song

sparrow blood smears. Furthermore, the amount of time between arrival and capture for

each bird is uncertain so it is unknown where an individual encountered their parasite.

The combination of not knowing where an individual became parasitized as well as the

relationship between parasite load and infection stage may explain why parasite load was

not related to migration distance.

Migration distance and immunity

My results provide partial support for the hypothesis that birds strategically

allocate immune function in relation to their migration distance as macrophage

phagocytosis decreased as migration distance increased for males but not females (Figure

3.6). This is opposite from my prediction that long-distance migrants should invest more

in innate immunity in response to their greater risk of encountering diverse and

unfamiliar parasites. Instead, this pattern supports the alternative hypothesis that the

energetic demands of migration may constrain the ability of migrants to invest in (at least

some aspects of) immune function. If long-distance migrants are not able to maintain

immune function while migrating, these individuals may be more prone to parasitism

(Møller et al., 2004) and aid in the long-distance transport of parasites. However, the

long-distance migrating males in the study population are likely young (migration

distance of males' increases with age) and because young age classes may participate in

migratory culling (see previous section), the long-distance transport of parasites may not

be a concern. Future research should investigate the flight performance and immunity of

parasitized birds in relation to sex, age, and prior migration distances before definite conclusions can be drawn as to which population classes are of greatest concern for the long-distance transport of parasites.

Other aspects of immunity (agglutination, circulating levels of IgY, and leukocytes) were not related to migration distance. While it is possible that birds were not caught soon enough after migration in order to fully capture the variation in immune function in relation to their migratory strategy, it is also possible that the distance a bird migrates does not influence these aspects of immunity suggesting that only birds in good physical condition will complete a migration (Hasselquist et al., 2007; Nebel et al., 2013). The vertebrate immune system is complex, such that different aspects of immunity may be unrelated to one another (Demas et al., 2011) and various immune functions may be differentially affected by migration distance.

The sex-specificity raises the question of why macrophage phagocytosis decreased with increasing migration distance for males but not females. A similar was observed in barn swallows where a measure of innate immunity decreased with arrival date in males but not females (Møller et al., 2004). As in most songbirds, male reproductive success in song sparrows requires establishing and defending territories (Nice, 1937). Consequently, the importance of early arrival on the breeding grounds is likely greater for males than for females in this species (Cristol 1995; Cristol et al., 1999). Because males themselves vary in migration distance, males migrating long distances may reduce time at stopovers to compensate for the greater distance travelled which may compromise energetic condition and immunity. Swainson's thrushes (*Catharus ustulatus*) and wood thrushes (*Hylocichla mustelina*) in poor energetic condition at stopover were

also in poor immunological condition and as the duration of migration stopover increased

T-cell mediated immunity also increased (Owen and Moore, 2008b). Such a scenario

would explain why males show reduced phagocytic capacity of macrophages with

increasing migration distance but females do not. However, this explanation assumes that

all males depart wintering grounds at the same time, regardless of their distance from

breeding grounds, and such a relationship is unknown.

Research to date has shown inconsistent relationships between immunity and

migration. For example, while innate immune function of European starlings (*Sturnus*

vulgaris) is compromised after flight in a wind tunnel (Nebel et al., 2012), red knots

demonstrate no effect of endurance flights on acquired immunity (Hasselquist et al.,

2007) and innate immunity is unaffected by endurance flights in western sandpipers

(*Calidris mauri*; Nebel et al., 2013). Additionally, innate immunity of red knots did not

vary during the stages of their annual cycle in which migration occurs (Buehler et al.,

2008); however, Swainson's thrushes (*Catharus ustulatus*) in migratory condition show

reduced cell-mediated immunity compared to control birds (Owen and Moore, 2008a).

Furthermore, Swainson's thrushes, wood thrushes, and veerys (*Catharus fuscescens*) had

lower leukocyte and lymphocyte counts when in migratory condition (Owen and Moore,

2006) and Swainson's thrushes, wood thrushes, veerys and gray-cheeked thrush

(*Cathatus minimus*) arriving at stopover in poor energetic condition had low leukocyte

and lymphocyte counts, though heterophil counts and IgG concentration were not related

to energetic condition (Owen and Moore, 2008b). The inconsistent findings in the

literature to date suggest that the extent to which immunity is affected by migration is

likely species- and condition-dependent, may vary depending on the specific immune

pathways investigated (Altizer et al., 2011), and the timeframe relative to migration in which immunity is measured. Regardless, my research is the first to link some aspect of immunity to individual migration distance, addressing how the migration distance of an individual may influence its innate and acquired immune function. Such information is important in order to evaluate how migratory individuals balance the energetic demands of migration with the parasite fauna they encounter.

4.3 Seasonal variation in parasites and immunity

Seasonal variation in parasitism

As predicted, parasite load and the incidence of infection with a blood-borne parasite increased as birds recovered from migration and progressed into the breeding phase of the annual cycle (inferred by capture date, Figure 3.7; Figure 3.8). As temperatures increased throughout the sampling period, insect vectors (mainly mosquitoes and blackflies) that transmit blood-borne parasites between hosts also increase in abundance at the breeding ground of my population (data from Peel, Ontario; Wang et al., 2011). Furthermore, the development rate of malarial parasites increases with temperature (Fialho and Schall, 1995). These conditions would increase the likelihood of transmission from host to host as the sampling period progressed and increase the incidence of parasitism in the population as a result. These conditions may also explain the increase in parasite load (per 10 000 red blood cells) in light of the relationship between infectious stage and parasite load (see migration distance and parasitism above). Chronic infections (low parasite load) persisting from migration were observed earlier in the breeding phase when birds are recovering from migration while

the incidence of acute infections [load > 15 infected cells, representing a peak in infection (Sarquis-Adamson and MacDougall-Shackleton, 2014, In Review)] appears to be related to vector emergence, as high intensity infections were only observed later in the breeding phase when vectors emerge (Wang et al., 2011). Because the incidence of infection and total parasite loads were greatest late in the breeding phase, and not once birds had arrived to the breeding grounds, this may suggest the risk of encountering parasites along migration may not be as great as previously anticipated. However, it is also possible that highly infected individuals may not survive the migration to breeding grounds (migratory culling; Bradley and Altizer, 2005) and those individuals are not present in the data (Section 4.2).

There were also sex-specific patterns in parasitism in relation to the phases of the annual cycle. The incidence of infection with a blood-borne parasite increased over the sampling period as birds transition into breeding condition for males but did not change over sampling period for females (Figure 3.8). Early in the breeding phase of the annual cycle, females are nest building (Nice, 1937) and as a consequence are flying around to gather materials. Later in the breeding season females are incubating eggs and are less conspicuous as a result, countering the increased prevalence of vectors later during this time. On the other hand, males are conspicuous through the whole season to maintain their territories through song (Nice, 1937). Singing, in comparison to sitting, has a higher basal metabolic rate, requires consuming more oxygen and, in turn, releases more CO_2, which mosquitoes are attracted to (Gillies, 1980; Ward et al., 2003). Because males maintain the same behavior through the sampling period, the increased incidence of infection for males may simply be due to the increased prevalence in vectors later in the

season when temperatures rise (Fialho and Schall, 1995). The increase in vector prevalence may not have the same effect on females as they are less conspicuous and produce less CO_2 than males during incubation.

Seasonal variation in immunity

As predicted, innate immunity decreased as birds progressed into the breeding phase of the annual cycle [macrophage phagocytosis (Figure 3.9), and proportion of monocytes and thrombocytes (Figure 3.11] while acquired immunity tended to increase (IgY; Figure 3.10). Red knots also experience an abrupt drop in phagocytic (innate) immune measures when transitioning to breeding condition (Buehler et al., 2008). This shift may be explained by the parasites birds encounter in relation to their annual cycle, based on the 'pace of life' hypothesis (Ricklefs and Wikelski, 2002). This hypothesis predicts that "fast-living" individuals (short lifespan, short development times, few large clutches) should invest in a general, non-specific immune response as they are less likely to re-encounter the same parasites throughout their lifetime. In contrast, "slow-living" individuals (long lifespan, long development times, many small clutches) should invest more in an acquired response which can "remember" previously encountered antigens, because such individuals are more likely to re-encounter familiar parasites throughout their lifetimes (Martin et al., 2007). Supporting this, immunity of house sparrows is related to their pace of life where antibody responses (acquired immunity) are greater in "slow-living" individuals (Martin et al., 2006). Similarly, the likelihood of re-encountering the same parasites may also vary with capture date. As my song sparrows remained on the breeding grounds (later date of capture), the chance of encountering a

familiar parasite would increase and that of encountering a novel parasite would decrease. Consistent with this expectation, innate immune function decreased through the season (Figure 3.9; Figure 3.11) and acquired immune function tended to increase throughout the breeding season (Figure 3.10), although not significantly so. However, the amount of time spent on the breeding grounds before their capture is unknown, my conclusions are speculative and further work should investigate the immunity of the same individuals through the sampling period.

Life-history theory predicts that the high energetic and nutritional costs associated with reproduction must be traded off against other energetically costly functions, such as immunity. The immunocompromising effects of reproduction coincide with the observed shift from innate to acquired immunity, and may enhance the decrease in innate immune function while hampering the increase in acquired immune function. This relationship may elucidate why the increase in acquired immune function was not significant while the decrease in innate immune function was. Female song sparrows captured late may be incubating, and reduced lymphocyte titres and specific antibody response (acquired immunity) are associated with increased incubation effort in female common eiders (*Somateria mollissima*; Hanssen et al., 2005). Such a decrease in acquired immune function during incubation may have hampered the observed increase of circulating IgY. This may explain why the predicted increase in acquired immune function throughout the season was not significant unlike the decrease in innate immune function. Moreover, the decrease in innate immune function through the sampling period would have only been enhanced by reductions in immunity during incubation.

Considering only one measure of acquired immune function was evaluated in this study and demonstrated possible trends, but not significance, it would be important in future work to investigate additional parameters of acquired immunity. However, few studies have achieved this under field conditions. Moreover, very little work has explored variation in acquired immunity in free-living animals. This is primarily due to logistic difficulties of quantifying acquired immunity in the field, in which reliably recapturing individuals is very difficult, and the simplest way to quantify immunity is to collect a single sample of blood from which plasma can be collected and later analyzed in a laboratory setting (Demas et al., 2011). In contrast, the best method to quantify acquired immune function in the field involves capturing and injecting individuals with a novel pathogen, not likely to be encountered in the wild; recapturing and re-injecting individuals with the pathogen after they have cleared the infection; then, within a restricted window of time, capturing individuals a third time to evaluate the specific antibodies produced in response to the pathogen (Demas et al., 2011). Repeatedly capturing free-living animals, especially within restricted periods of time, is often unrealistic. As a result, acquired immunity in free-living animals is often studied by alternative methods such as quantifying circulating levels of immunoglobulins. While immunoglobulin levels can be an informative assay, the fact that immunoglobulin levels fluctuate after infection means that the assay may be biased by how recently an individual has encountered a parasite (Atkinson et al., 2001).

It should be noted that observed decrease in innate immune function [macrophage phagocytosis (Figure 3.9), and proportion of monocytes and thrombocytes (Figure 3.11)] was greatest early in the breeding phase when birds were recently returned, and

presumably recovering, from migration. Similar results have been found in captive red

knots which show a decrease in phagocytic activity while transitioning into spring

migratory condition (Buehler et al., 2008). During migration, birds are less likely to re-

encounter the same parasite multiple times and are more likely to encounter novel

parasite in comparison to when they remain in the same place (Møller and Erritzøe,

1998). Thus, during migration birds should benefit from investing in a general response

which does not discriminate amongst antigens, i.e. innate immunity. Consistent with this,

a measure of innate immunity, phagocytic activity, was greatest early in the sampling

period when birds were recovering from migration. These findings may suggest that

innate immune function may be an important parameter of immunity during migratory

journeys.

4.4 Conclusions

While previously reported across species (Møller and Erritzøe, 1998; Figuerola

and Green, 2000), my research is the first research to demonstrate that migration distance

may affect the incidence of infection within a population. While sexes did not differ in

infection with a blood-borne parasite, there were interesting patterns of variation between

age classes in the incidence of infection in relation to migration distance. Within adults,

the incidence of infection increased with migration distance, suggesting that, within a

population, birds migrating longer distances may be more likely to encounter parasites

throughout their lifetimes than resident or short-distance migrants. However, the

incidence of infection was not related to migration distance for young birds. This may be

because only the young birds making short migrations are able to avoid, tolerate, or clear

infection prior to arrival on the breeding grounds, suggesting there may be differential migratory culling (Bradley and Altizer, 2005) in this population of song sparrows where young birds, but not adults, may lower infectious disease risk by removing infected individuals from the population. This adds to our understanding of how parasites may act as an important selection pressure in shaping migratory behaviour of birds (Bradley and Altizer, 2005; Møller and Szép, 2011) and in young birds for either a high tolerance of infection (Altizer, 2011) or enhanced immunity (Davidar and Morton, 1993).

Since the suggestion that migratory birds encounter a greater diversity of parasites (Møller and Erritzøe, 1998), the ways in which immunity and migration interact and trade-off have been of great interest (Møller et al., 2004; Owen and Moore, 2006; Hasselquist et al., 2007; Owen and Moore, 2008a, 2008b; Altizer et al., 2011; Nebel et al., 2012). I report the first investigation of immunity in relation to individual variation in migration distance within a species. I demonstrate that migration distance is negatively associated with a costly phagocytic innate immune response in male song sparrows. Males themselves vary in migration distances and, because males suffer greater fitness consequences for late arrival (Cristol, 1995; Cristol et al.,1999), males that migrate long distances may make rushed migrations to compensate for the greater distance they need to cover. As a result, these males may be poor condition and suffer immunosuppression (Owen and Moore 2008b).

I also found evidence that the incidence of infection varies according to the annual cycle where both the incidence of infection for males and the parasite load for both sexes increased as birds recovered from migration and shifted to breeding condition. Such relationships are likely driven by the abundance of vectors also increasing as spring

progressed (Wang et al., 2011). The variation in the incidence and total load of parasitism in relation to the annual cycle may have direct effects upon song sparrow immunity. I found a possible immunological shift from innate immunity to acquired immunity. Such a shift may be related to the familiarity of parasites they are likely to encounter where as birds remain on the breeding grounds the likelihood of encountering a familiar parasite likely increases while that of a novel parasite may decrease. Furthermore, the distinct drop in innate immunity as birds recover from migration and transition to breeding condition suggests that innate immune function may be important during migratory journeys (Buehler et al., 2008).

In all, phases of the annual cycle have consequences in terms of interactions with familiar or unfamiliar parasites and, in turn, an individual's immunocompetence (Altizer et al., 2011). Such relationships have been well studied across species but less so within a species. I report, for the first time, an investigation of how variation in migration distance of a differentially migrant population may affect parasitism and immunity at the individual level. Such investigations are important in light of anticipated range expansions by parasites and their vectors (Parmesan, 2006) and the concern of migratory birds as long-distance transporters of infectious disease.

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6   Appendix

Appendix A   Phagocytosis assay protocol

Protocol adapted from Millet et al. (2007) for smaller quantities of blood. This assay examines the phagocytic activity of macrophages through quantification of percent of fixed macrophages that have engulfed at least one fluorescently labeled particle.

Step 1: Reconstitution of BioParticles according to manufacturer's instructions

Reagents and Supplies:

- BioParticles® *Escheria coli* BODIPY FL 505/513
- BioParticles® *Staphylococcus aureus* BODIPY FL 505/513
- Sodium azide 100 mM (NaN_3)
- 0.01M phosphate buffered saline (PBS)

1. Reconstitute particles using tissue grade PBS with 2mM Sodium Azide (NaN_3) to 20mg ml^{-1}. This should be done in a hood using sterile techniques.

    a. Prior to reconstitution, pellets are desiccate and stored at -20°C. Should be protected from light (wrap glass vial in Aluminum foil).

    b. Pellets are at 10mg: add 500ml of PBS with 2mM NaN_3

        i. Add 490μl PBS (4°C) and 10μl 100mM NaN_3 (4°C) to glass vial with desiccate pellet.

2. Vortex reconstitution 3 x 15 seconds at highest setting.

3. Sonicate the particles 3 x 20 seconds.

4. Particles should be at $\approx 3x10^8$ particles mg^{-1} in original solution: count if needed using a hemocytometer.

5. Reconstitution can be stored for up to two months at 4°C. Store in light proof container to protect fluorescence (wrap in foil).

Step 2: Making Cell Media

Reagents and Supplies:

- CO_2-Independent Media
- Penicillin-steptomycin
- L-glutamine 200 mM
- Fetal Bovine Serum

Protocol:

For 50ml total volume:

1. Pour approximately 50ml media (Gibco, CO_2 independent medium 1X, without L-glutamine) in a 50ml tube.
2. Using a serological pipette, remove 46ml media into another 50ml tube.
3. Add 2.5ml fetal bovine serum.
4. Add 1ml of 4mM L-glutamine.

    a. Add 1 mL of 200 mM L-glutamine to 49 mL of cell media for a final concentration of 4 mM L-glutamine (makes 50 mL 4 mM L-glutamine).

5. Add 0.5 ml penicillin streptomycin.
6. Mix.
7. Pour into four or five 15ml tubes.
8. Cap and store at 4˚C.

Note: Cell media varies in the amount of time it lasts. Growth may occur in a vial – be

sure to check for this before use! If going away for a field season, ensure you bring

supplies to make more.

Step 3: Blood Collection

1. Use sterile blood collection techniques.

2. Aim start running assay within 40 minutes of blood collection.

Step 4: Phagocytosis Assay

Reagents and Supplies:

· Nunc Lab-Trek chamber slide (8 well)

· Microcentrifuge tubes

· Bioparticle reconstitution

· Cell media

· Methanol

Protocol

1. TURN ON INCUBATOR TO 41˚C.

2. Remove blood from capillary tube by scoring off clay end with a small file.

   Empty cap tube into a sterile microcentrifuge tube. May need to suck blood out of

   cap tube using a pipetman, or softly blow blood out scored end using a pipettor.

3. Dilute blood to 1:20 in sterile cell media to final volume of 200μl.

   a. Add 10μl of blood to 190μl cell media.

4. Prepare bioparticle dilution in cell media

   a. Will be adding a total volume of 75.75μl of bioparticle dilution per well.

b. For E. coli, need to have a 1:100 WBC:particle ratio. Adding 20µl of 1:20 diluted blood per well gives ~ $1.516x10^4$ WBC per well (~758 WBC's per µl of diluted blood). Therefore, with 1:100, when using 20µl of diluted blood per well will need to have $1.516x10^6$ E. coli particles per well. There is $6x10^6$ particles per µl. Thus will need 0.25µl E.coli stock per well for proper cell:particle ratio.

    i. 0.25µl E.coli stock x 9 wells (allows excess) = 2.25µl per slide

    ii. 75.5µl cell media x 9 wells (allows excess) = 679.5µl per slide

c. **ADD 680µl cell media and 2.25µl stock E. coli to microcentrifuge tube (for one slide).**

d. For S. aureus 1:200 WBC:particle ratio. When using 20µl of diluted blood per well will need to have $3.032x10^6$ particles per well. Thus will need 0.51µl S. aureus stock per well.

    i. 0.51µl S. aureus stock x 9 wells (allows excess) = 4.6µl per slide

    ii. 75.50µl cell mediea x 9 wells (allows excess) = 679.5µl per slide

e. **ADD 677µl cell media and 4.6µl stock S. aureus to microcentrifuge tube (for one slide).**

5. Pipet diluted blood up and down to create an even suspension.

6. Pipet 20µl into each well of the slide.

7. Mix bioparticle dilutions by inverting the tube a few times prior to pipetting.

8. Pipet 75.75µl into each well

   a. Be sure to minimize the amount of time that fluorescent particles are in the
      light. Work in minimally lit conditions and cover tubes with aluminum
      foil.

9. Incubate at 41˚C for 15 minutes.

10. Remove from the incubator and place on ice for 5 minutes to end phagocytosis.

11. Wash 2x gently with 91µl of 4˚C cell media. Slowly let the media wash down the
    side of the well as to keep cells fixed to the slide.

12. Tilt the slide so the solution flows to the chamber corners. Remove the media,
    pipetting from the corner of the well and avoid touching the bottom of the slide.

13. Add 91µl of -20˚C CH_3OH (methanol) and place on ice for 5 minutes. The
    methanol should be added gently to the side of the well.

14. Remove methanol using the same techniques as for removing the media washes.

15. Remove the plastic wells from the slide and place the slide in a light proof
    container (slide box) until microscopy.

Step 5: Microscopy

Expect a few hours of practice to identify cell types. If you have a spare slide practice on
it to avoid damaging the fluorescence of particles on important slides. It can also help to
look at a few blood smears and get familiar with the different cell types before looking at
these slides.

What am I counting? You want to count the number of adherent cells (macrophages,
WBCs) that have engulfed at least one fluorescently-labelled bacteria particle. You do
not need to count the number of particles the cell has engulfed, just whether or not the
cell has engulfed one (Figure 6.1). Convert the "score" of each well to a percent (percent

of cells that have engulfed at least one particle) and then average all of the wells (E. coli and S. aureus separately) to get a final average for the bird.

How many adherent cells to count? 100-125 per well should suffice for a total of 400-500 cells counted. Sometimes (depending on how vigorous washes are) you may not find enough cells; go with what you have.
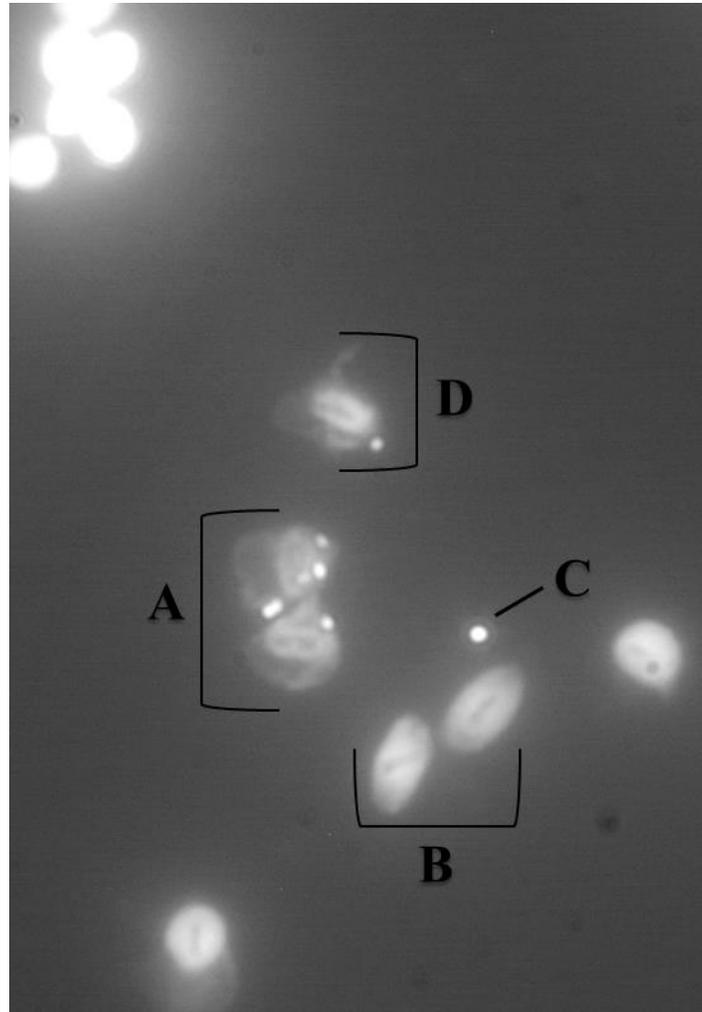
Figure 6.1 Flourescence microscopy (excitation/ absorbance spectrum of 505/513) of a phagocytosis assay slide. A identifies two leukocytes, both of which have engulfed at least one fluorescent particles; B identifies two avian red blood cells; C identifies a fluorescent particle; D identifies a damaged red blood cell.

Appendix B   Hemolysis and hemagglutination assay protocol

Protocol adapted from Matson et al. (2005) to quantify two interrelated humoral components of innate immunity: natural antibodies (NAbs) and the complement enzyme cascade, both of which are associated with innate immunity. This assay requires 50 µL of plasma but can also be run using 40 µL of plasma *if* plasma is limited.

Materials and Equipment:

- 37^oC incubator

- Eppendorf centrifuge

- Micro-hematocrit centrifuge

- Flatbed, positive transparency scanner (EPSON Perfection 4990 Photo)

- 45° inclination (e.g. slanted Pipette rack)

- Pipettes: P-1000, P-100, and multi-channel pipette OR repeater (to draw 25 µL)

- Pipette tips: 1000 µL and 200 µL

- Heparinized micro-hematocrit capillary tubes and clay

- Parafilm

- Microcentrifuge tubes: 0.5 mL and 1.5 mL

- 50 mL Falcon tubes

- Plastic loading trays

- 96-well U bottom microplates Corning#3788

- Chicken Plasma

- Whole rabbit blood

- 0.01M phosphate buffered saline (PBS)

Step 1: Solution preparation

PBS

5.7g dibasic sodium phosphate

1.2g monobasic sodium phosphate

4.5g NaCl

1. Add all ingredients into a 500 mL beaker.

2. Bring up to 500 mL with deionized water.

3. Stir until dissolved

4. Store at 4 °C.

Chicken plasma

1. Reconstitute with PBS according to volume purchased.

    a. Add 1 mL PBS per 1 mL freeze-dried chicken plasma.

2. Vortex 3x for 20 seconds each.

3. Divide reconstitution into 220 µL aliquots in 600 µL eppendorf tubes.

4. Store plasma at -20 °C.

1% Rabbit red blood cell solution

1. Invert the rabbit RBCs gently to ensure it is well mixed.

2. Pipette approx. 1-1.5 mL of RBCs into a 1.5 mL Eppendorf microcentrifuge tube.

3. Centrifuge the rabbit RBCs at 275 x g for 5 mins.

4. Wash RBCs three times.

    a. Remove the supernatant with a pipette.

    b. Resuspend with PBS to approx.. 1 mL

> * Can refill with more RBC to end up with higher hematocrit and, in turn, more 1% solution. Only do this once (defeats the purpose of washing otherwise)

    c. Centrifuge   (Wash #1)

    d. Repeat a. – c. (Wash #2)

    e. Repeat a. – c. (Wash #3)

    f. Resuspend with PBS to approx. 1 mL [Resuspend (R)]

> * Refer to 'example sample sheet' for checklist of these steps.

5. Fill 2 heparinized microcapillary tubes with the resuspension (~75% full).

6. Centrifuge the microcapillary tubes in a micro-hematocrit centrifuge at 13 000 rpm or maximum speed for 5 minutes.

7. Calculate hematocrit of each microcapillary tube using a hematocrit reading card or calculate the percent of packed RBCs. Average hematocrit of both tubes.

8. Use the average hematocrit to calculate the dilution to make a 1% solution.

    a. If the average hematocrit is 23% then add 1 mL of resuspension to 22 mL PBS.

9. Make as many 1% rabbit blood solutions as possible in 50 mL Falcon tubes and store in the fridge (4 ˚C) until needed.

<center>** Prepare a fresh batch every 3 days**</center>

Step 2: The assay procedure

Note: These procedures are for volumes of 25 μL. If you are using 20 μL of plasma, change all the pipetted volumes from 25 to 20.

1. Because the assay is time-sensitive, ensure you have a scanner that will take proper scans (top-down) AND THAT YOU KNOW HOW TO USE IT AND SAVE FILES.

2. Before you start, bring the PBS and rabbit blood out of the fridge, and the chicken plasma out of the freezer and allow them to warm up to room temperature (~ 30 minutes).
   TURN ON THE INCUBATOR AND SET IT TO 37°C.

3. Label two plastic loading trays for the multi-channel pipettor: one for PBS and the other for the 1% rabbit blood solution.

4. Label the bottom right hand corner of the plate, e.g. year, month, day, and the plate letter (e.g. 20060608A). This will be useful to distinguished plates; record this on the 'example sample sheet'.

5. Vortex chicken plasma well (5-10 seconds). Pipette 25 µL of chicken standard into row 1 and 2 of column H and A. These columns will serve as your positive control from plate to plate.

6. Vortex your plasma samples well (5-10 seconds). Pipette 25 µL of plasma into rows 1 and 2 of each column so that rows B through G each contain a unique plasma sample. Record the sample ID's in the 'example sample sheet'.

7. Using the multi-channel pipette, pipette 25 µL of PBS into all columns (H to A) from rows 12 to 2.

8. The serial dilution:

   a. To begin the serial dilution, mix the contents of row 2 well.

   b. Place the tips of the multi-channel pipettor on an angle in the liquid to avoid scratching the bottom of the plate. Suck up the liquid, then expel it

gently. Do this approximately 4 times. Do your best to avoid creating any

bubbles in the concoction (may interfere with scoring).

   c. Pipette 25 µL from row 2 and transfer into row 3.

   d. Mix the contents of row 3 by gently sucking up the liquid, then expelling

      it gently back into the wells.

   e. Pipette 25 µL and transfer into row 4. Continue down the rows until you

      reach row 11.

   f. In row 11 pipette 25 µL, mix the contents, and discard. <u>ROW 12 HAS **NO**</u>

      <u>DILUTED PLASMA - only 25 µL PBS (this serves as the negative control).</u>

9. Using the multi-channel pipettor, add the 1% rabbit RBCs (now timing counts)

    into each well.

10. Carefully swirl the plate horizontally (in circular motion on the lab bench) to mix.

11. Seal the plate with Parafilm and cover with the plate lid.

12. Incubate the plate for 90 min at 37°C and record time on the 'example samples

    sheet'.

13. After 90 min remove plate from the incubator.

14. Incubate the plate for 20 minutes at room temperature at a 45° inclination (long

    axis) by placing it on the pipettor rack.

15. At 20 min scan the plate with a flatbed scanner.

   a. Crop the preview to have only the plate scanned.

   b. Save as a "TIFF" file.

   c. Label the plate: year-month-day-plate-incubation time (for example

      20060608A20).

16. Remove the plate from the scanner. Leave the plate flat on the lab bench at room temperature for 70 min.

17. After 70 min scan the plate again.

    a. Crop the preview to have only the plate scanned.

    b. Save as a "TIFF" file.

    c. Label the plate: year-month-day-plate-incubation time (for example 20060608A90).

18. Remove the plate from the scanner. At this point you are done with the plate. You can stack them in the sink for cleaning.

Step 3: Scoring agglutination and lysis

Sample Organization

1. Crop all scans by row. Scans of both aggluintation and lysis need to be cropped.

2. Save cropped samples by row (e.g. 20060608A20-A, 20060608A20-B, etc.).

3. Create an Excel file for scoring.

Scoring agglutination (natural antibodies)

With reference to Figure 6.2:

· Agglutination scores range from 0-11.

· Compare each well bottom up starting from the negative control (row 12) and count the wells that have an oblong (dripping) similar to the negative control from gravity when the plate was titled.

· Compare each well top down starting from the positive control and count the wells that have agglutination that is completely intact. The last well that is
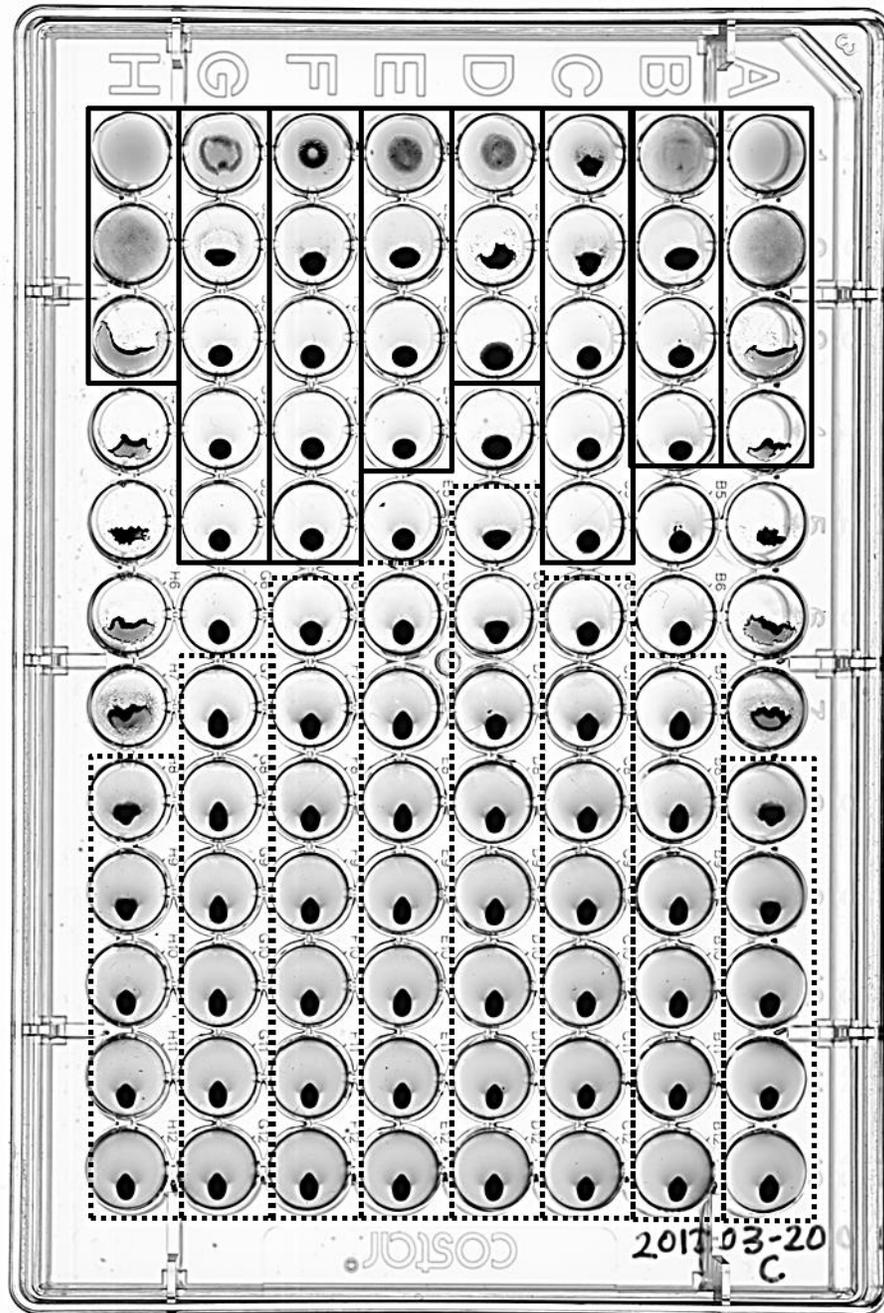
Figure 6.2 A hemolysis-hemagglutination microplate after incubating at a 45° incline for visual scoring of agglutination. Solid squares represent agglutination that is completely intact. Dotted squares represent wells that have oblong (dripping) similar to the negative control (row 12). Columns have the respective scores: A=6, B=5.5, C=5, D=3.5, E=4.5, F=5, G=5.5, H=5.5.

fully intact is the agglutination score. If the next well is partially intact, that well can receive a score of 0.5.

- · For example, if wells 12 through to 8 are oblong these are negative. If well 1, to 6 are agglutinated these are positive. If well 6 is completely intact, but well 7 is partially intact and partially oblong, then the agglutination score would be 6.5.

- · If there are several wells that are intermediate (not completely intact but also not oblong) take the mid-point of the intermediate wells as the score for agglutination.

- · For example, if wells 4 to 6 are intermediate, then (4+6)/2 is the agglutination score (5).

Scoring lysis (complement activity)

With reference to Figure 6.3:

- · Lysis scores range from 0-7

- · Start by comparing each well from the positive control down to look for lysis.

- · If the plasma in a well is 75 % lyzed (or greater), score = 1.

- · If the plasma in the well is >30 % but <75%, score = 0.5.

- · For example, if wells 1, 2 are fully lyzed and well 3 is greater than 75 %, than the lysis score is 3.

- · If there are several wells that are intermediate, take the mid-point same as above.

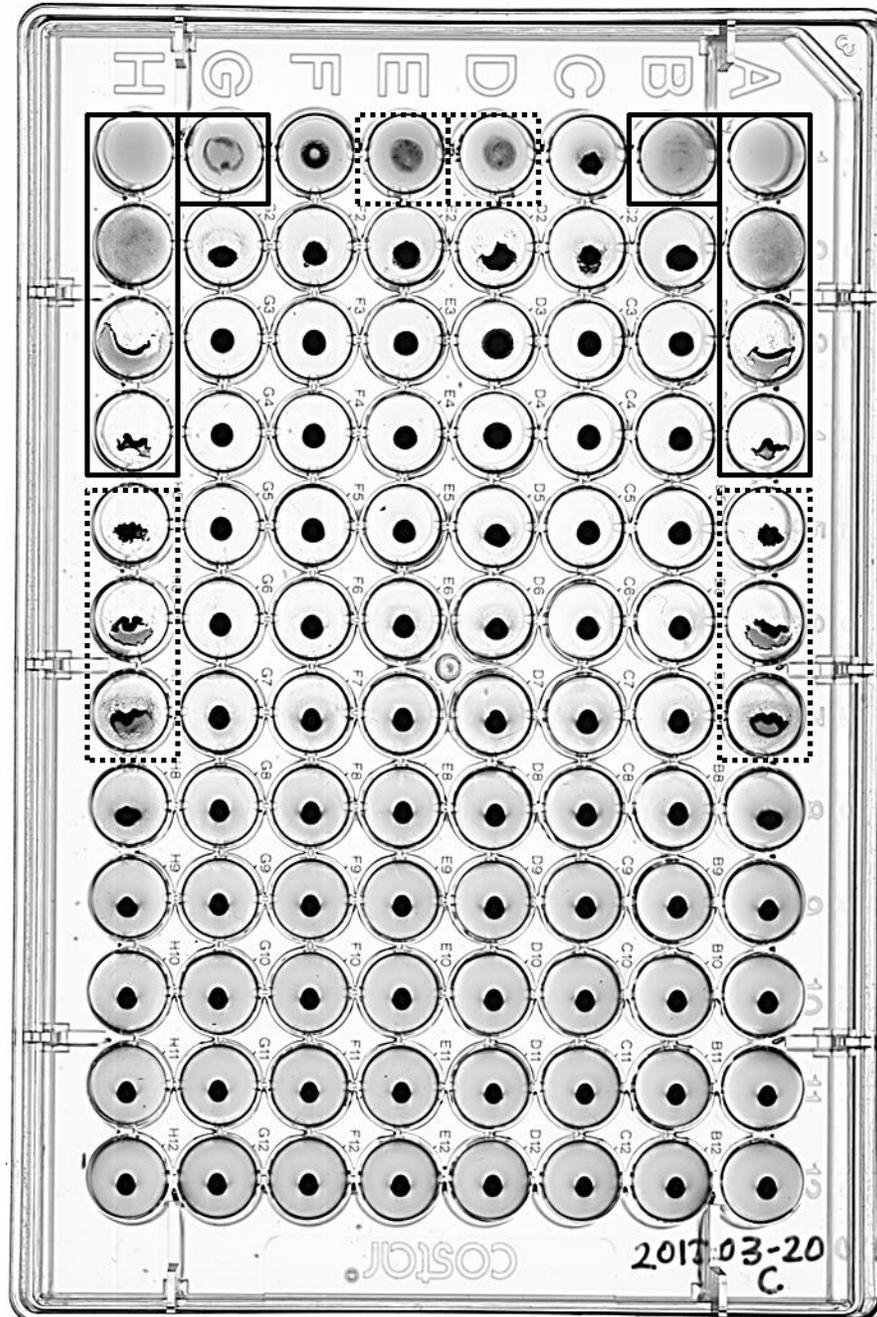**Figure 6.3** A hemolysis-hemagglutination microplate after incubating flat for visual scoring of lysis. Solid squares represent < 75 % lysis. Dotted squares represent that are > 30 % lyzed but < 75 %. Columns have the respective scores: A=6, B=1, C=0, D=0.5, E=0.5, F=0, G=1, H=6.

Appendix C   ELISA immunoglobulin Y (IgY) assay protocol

Protocol is used to evaluate the circulating concentration of IgY in the blood (acquired immunity).

Note: This protocol is set for IgY, but the procedures can really be used to measure anything. If you wish to measure IgG, for example, you would just need to order the anti-IgG chicken antibody from Sigma.

Materials and Equipment:

- Incubator
- Pipettes: P-1000, P-200, P-10
- Multi-channel pipette or repeater
- Pipette tips: 1000 μL and 200 μL
- Parafilm
- 15 mL Falcon tubes
- Plastic loading trays
- 96-well flat bottom microplates Corning #3596
- Sodium carbonate (Na_2CO_3)
- Sodium bicarbonate ($NaHCO_3$)
- Citric acid anhydrous ($C_6H_8O_7$)
- Sodium phosphate (Na_2HPO_4)
- Tween 20
- Sodium Chloride (NaCl)
- Potassium phosphate monobasic (KH_2PO_4)
- Hydrogen peroxide 31 % (H_2O_2)

- 2,2-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS; $C_{18}H_{24}N_6O_6S_4$)

- Anti-chicken IgY

- Fat free skim powdered milk

- 0.01 mM phosphate buffered saline (PBS)

Step 1: Solution preparations

Should be done the day prior to running the assay. Before beginning your solution

preparation, make sure that you have calibrated and zeroed the pH meter (if using one).

Instructions on how to do this are in the manual for the pH meter. Otherwise, pH paper

works as well.

Dilution Solution

1. Make 0.25 L of 0.1M Na_2CO_3:

    a. Add 2.65 g of Na_2CO_3 to 250 mL deionized water in a 250 mL beaker.

    b. Cap and mix.

2. Make 0.5 L of 0.1 M $NaHCO_3$:

    a. Add 4.2g of $NaHCO_3$ to 500 mL deionized water in a 500 mL beaker.

    b. Cap and mix.

3. Add the Na_2CO_3 solution slowly to the $NaHCO_3$ solution until a pH of 9.6 is

    reached.

4. Store in 4 °C fridge. Solution will last for months.

PBS-Tween solution

1. Make 8L of PBS Solution. First make the following three solutions separately:

    a. Add 35.06 g of NaCl to 4 L of deionized water.

    b. Add 9.59 g K_2HPO_4 0.96 L of deionized water.

    c.   Add 64.73 g of Na_2HPO_4 to 3.04 L of deionized water.

2. Mix all of the above solutions to get 8 L of PBS.

3. Add 4mL of Tween to the PBS using a pipetman– it is thick and you will not be able to pipet it with a typical pipettor like a P1000 or P5000.

4. Store in +4 °C fridge. Solution will last for months.

Milk solution

1. For 1 assay (or plate): mix 0.75 g of powdered milk in 15 mL of PBS-Tween solution.

2. Do not store, make as needed.

Antibody Solution

1. For 1 assay (or plate): mix 44 µL of chicken anti-body with 11 mL of PBS-Tween.

2. Store in 15 mL Falcon tubes. Date and label the tubes with solution name.

3. Store pre-prepared tubes in -20 °C freezer. Unfreeze as needed.

Revealing solution

1. Make 0.1M citric acid: Add 9.607 g to 500 mL of deionized water.

2. Make 0.1 Na_2HPO_4: Add 17.907 g to 500 mL of deionized water

3. Carefully add 0.1M citric acid to 0.1M Na_2HPO_4 until you reach a pH of 5.

    Note: 500 mL of citric acid is not enough to bring the pH down to 5. You have two options: make 1L of citric acid and add to 500 mL of Na_2HPO_4 or make 500 mL of each solution, but add 500 mL of citric acid to 250 mL of Na_2HPO_4, depending on which you feel more comfortable with.

4. Add 0.05 g of ABTS [2,2-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid)] to 100 mL of the above solution.

5. Prepare frozen samples by measuring out 11mL of the mixture containing the ABTS in labelled 15 mL Falcon tubes and store in -20 °C freezer. Unfreeze as needed.

6. For one assay (one plate): Unfreeze one sample of 11 mL of the ABTS revealing solution and add 11 µL of the hydrogen peroxide. Mix well (i.e. vortex 5-10 seconds) before use.

Step 2: Standardization

Before you begin measure the immunoglobulin content of blood plasma, you must determine the appropriate dilution of plasma according to the species being studied. To do so, you must work with a range of plasma dilutions from 1/500 to 1/64000.

1. To test for the necessary dilution, begin by diluting 12 plasma samples at 1/500 (2 µL plasma + 1000 µL of dilution solution) and place 200 µL of this diluted plasma in the first row (row A) of your Elisa plate (ALWAYS BE CAREFUL NOT TO TOUCH THE BOTTOM OF THE PLATE WITH YOUR FINGERS; wear gloves at all times when handling your plate).

2. Fill the rest of the rows (rows B through H) with 100 µL of the dilution solution (free of any plasma samples).

3. Next, using the 8 tip multi-channel pipettor, take 100 µL from row A and add it, while mixing in circular motions, to row B. Then take 100 µL of row B and add it will mixing in circular motions to row C. Continue doing this, making sure that

the diluted plasma is well mixed. Once you have added the 100 μL to row H, remove 100 μL and discard it. Dilutions should follow the setup depicted in Figure 6.4.

4. Follow the procedure outlines in Step 3.

5. You will then need to calculate the average quantity of immunoglobulin for the 12 samples for all of the dilutions and plot these data as a graph with X = dilution and Y = immunoglobulin average.

6. The dilution you should use will be the one where the slope of this graph is steepest (Figure 6.5; Figure 6.6).

Step 3: The assay

This procedure is to be completed in 2 days. The first day will require about 2 hours while the second will require about 5-6 hours. If you do not want to be in the lab forever, I suggest getting your plates into the fridge by no later than 11am the first day. You will be able to test 47 samples per plate. Do not touch the bottom of the plate with your bare fingers!! To avoid this, wear gloves at every single step you are handling a plate.

Day 1: Dilute blood plasma

1. SET INCUBATOR TO 37°C.

2. Using a beaker, pour out that day's aliquot of dilution solution. Allow it to warm to room temperature (~30 minutes).

3. Dilute the plasma using the serial dilution data you have generated for your species, or use a dilution from Table 6.1.

4. If you have n samples to perform, label n tubes with one coloured pen. Fill n tubes with 1000μL of dilution solution.
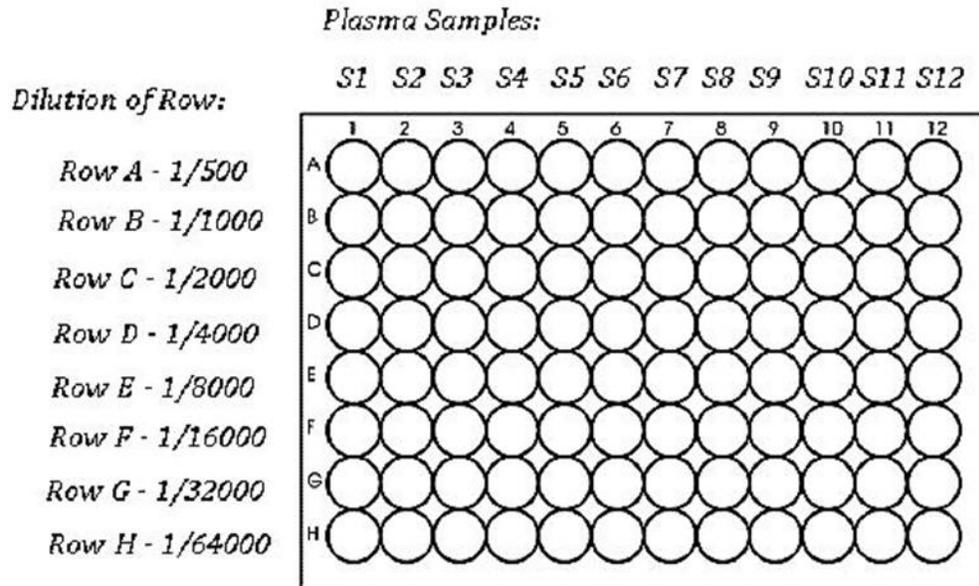
Figure 6.4 Depicts a dilution cascade setup in a 96-well microplate in order to evaluate the standardization appropriate for a species. Columns 1 through 12 are filled with unique plasma samples from the same species (S1 – S12). Rows increase in dilution from 1/500 (2 µL plasma + 1000µL of dilution solution) to 1/64000 (0.000015 µL plasma + 1000µL of dilution solution).
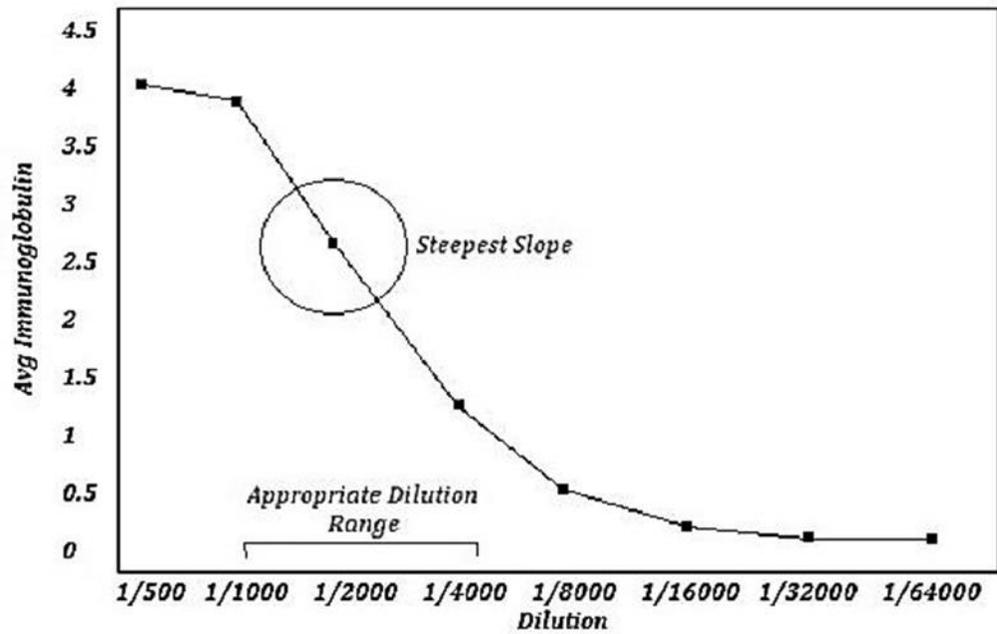
Figure 6.5 Average immunoglobulin concentration as a function of plasma dilution. Used to determine appropriate dilution for IgY assay. Circle depicts the steepest slope between dilutions and thus the appropriate dilution for the species.
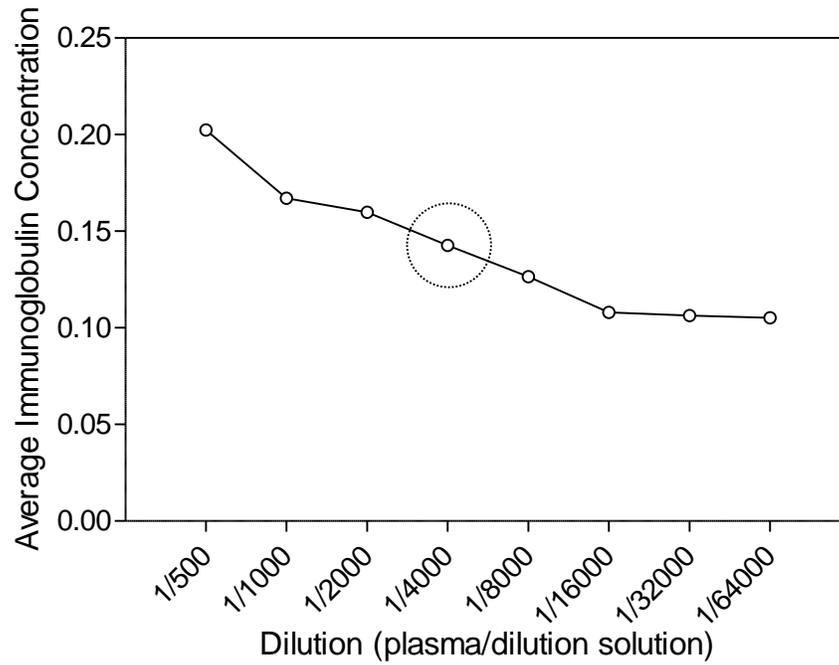
Figure 6.6 Average immunoglobulin concentration as a function of plasma dilution for song sparrows caught near Newboro, ON, in April – May 2013. Dashed circle depicts the appropriate dilution for song sparrow plasma.

Table 6.1 Table as reference for various species dilutions; proper dilution of plasma for the IgY assay and their respective dilution steps in order to obtain the final appropriate dilution.

Species	Final dilution (2 steps)	1st dilution step	2nd dilution step
Starling (adult)	1/8000	2µL plasma + 1000µL of dilution solution	62.5µL of 1st dilution + 937.5µL of dilution solution
Starling (chick)	1/4000	2µL plasma + 1000µL of dilution solution	125µL of 1st dilution + 875µL of dilution solution
Eider (adult)	1/32000	2µL plasma + 1000µL of dilution solution	15.65µL of 1st dilution + 984.35µL of dilution solution
King penguin (adult)	1/32000	2µL plasma + 1000µL of dilution solution	15.65µL of 1st dilution + 984.35µL of dilution solution
Zebra finch; Ring-billed gull chick	1/8000	2µL plasma + 1000µL of dilution solution	62.5µL of 1st dilution + 937.5µL of dilution solution
Song Sparrow	1/4000	2µL plasma + 1000µL of dilution solution	125µL of 1st dilution + 875µL of dilution solution

5. Add 2μL of plasma (vortexed) to each tube, making sure to change the pipette tip for every sample.

6. Close all tubes. Be sure to record which plasma sample (band number, code number, or what have you) corresponds to which tube number (using 1, 2, 3 etc.).

7. Depending on which dilution you are using, label a second set of tubes with a different coloured pen. Fill this second set of tubes (using n tubes if you have n samples) with the required amount of dilution solution (e.g. for adult starlings, this would be 937.5 μL). Ensure you know which colour pen is which dilution.

8. <u>VORTEX ALL SAMPLES OF YOUR FIRST DILUTION!!</u> Do not forget to do this, otherwise you will *have to start over.*

9. Add the required volume of the first dilution sample to the tubes you have prepared in step 5 (e.g. For starling adults, add 62.5 μL of the first dilution to the tubes you prepared containing 937.5 μL of dilution solution).
   Keep step 5 dilutions as a precaution in case you lose or make mistakes with the step 9 dilutions. This way you can just remake a step 9 dilution using what is left from step 4.

10. Vortex your diluted plasma samples for 5-10 seconds. Make sure you are using the samples which have been diluted twice, from step 9 in the previous section.

11. Place 100 μL of the first diluted plasma sample in the first well of the plate, as well as in the second well.

12. Keep filling the wells in order with 100 μL of diluted samples, making sure you have 2 wells for every sample.

Note: You should keep a guide regarding sample locations and load the samples in tube order (e.g. 1, 2, 3) so that you can trace your samples back to each bird.

13. Once you have filled in your 47 samples, fill the last two wells of the plate with 100 μL pure dilution solution (this will be your control).

14. Cover the plate with Parafilm (ensure that the parafilm stretches and covers each well) and place the lid on top of the parafilm.

15. Place for 1 hour in the incubator at 37°C.

16. After incubation, place the plate overnight in the 4°C fridge.

Day 2: Adding antibodies and revealing solution

1. In a beaker, pour out that day's aliquot of PBS-Tween and let it warm to room temperature (~ 30 minutes). Have a plastic loading tray ready (label it PBS-Tween) and pour into the tray once the solution has warmed to room temperature.

2. Before beginning any steps, label a plastic loading tray with Milk solution. Then proceed to make the milk solution (in a small beaker) and pour into the plastic loading tray. Cover with parafilm and leave on lab bench.

3. Turn on your incubator and set it for 37 °C.

4. 24 hours after leaving the plate in the fridge (just be consistent for all your samples) empty the wells by forcefully shaking the liquid out of the wells over the sink. Do this with gloves on to prevent fingerprints on the underside of the plates.

5. Once the wells have been emptied, blot the plate upside down on paper towels 3 times to get rid of any remaining liquid.
   Note: You do not want to leave the wells exposed to air, so proceed to next step rapidly.

6. RINSE 1: Using the multi-channel pipettor, place 200 µL of PBS-Tween solution in each well – this rinses the plate so you do not need to be super careful with volumes but you must make sure that you do not scratch the walls of the wells with the pipette tip.

7. Take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink and blot on a piece of paper towel 3 times to get rid of any remaining liquid.

    Note: You do not want to leave the wells exposed to air, so proceed to next step rapidly.

8. RINSE 2: Repeat rinse method in step 6 and empty as described in step 7.

9. Add 100 µL of the milk solution to each well using a multi-channel pipettor.

10. Cover the plate with parafilm and place the lid on top.

11. Put the plate in the incubator for 1 hour at 37 °C.

12. While the plate is in the incubator, defrost your antibody solution. Label another plastic loading tray "antibody".

13. After the incubation is over, take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink and blot on a piece of paper towel 3 times to get rid of any remaining liquid.

    Note: You do not want to leave the wells exposed to air, so proceed to next step rapidly.

14. RINSE 1: Using the multi-channel pipettor, place 200 µL of PBS-Tween solution in each well – this rinses the plate so you do not need to be super careful with volumes but you must make sure that you do not scratch the walls of the wells with the pipette tip.

15. Take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink and blot on a piece of paper towel 3 times to get rid of any remaining liquid.

    Note: You do not want to leave the wells exposed to air, so proceed to next step rapidly.

16. RINSE 2: Repeat rinse method in step 14 and empty as described in step 15.

17. Invert antibody solution gently to mix and pour into plastic loading tray. Using the multi-channel pipettor, add 100 µL of the antibody solution into each well. Be careful not to scratch the bottom of the plate with the pipet tips.

18. Cover with parafilm and place the lid on top. Put the plate in the incubator for 2 hours at 37 °C.

19. While the plate is in the incubator, prepare the revealing solution. Defrost the revealing solution on the bench. Label another plastic loading tray "Reveal".

20. Just before the plate is about to come out, add the 11µL of hydrogen peroxide and mix well before pouring into the loading tray.

21. After the incubation is over, take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink and blot on a piece of paper towel 3 times to get rid of any remaining liquid.

    Note: You do not want to leave the wells exposed to air, so proceed to next step rapidly.

22. RINSE 1: Using the multi-channel pipettor, place 200 µL of PBS-Tween solution in each well – this rinses the plate so you do not need to be super careful with volumes but you must make sure that you do not scratch the walls of the wells with the pipette tip.

23. Take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink and blot on a piece of paper towel 3 times to get rid of any remaining liquid.

    Note: You do not want to leave the wells exposed to air, so proceed to next step rapidly.

24. RINSE 2: Repeat rinse method in step 22 and empty as described in step 23.

25. Using the multi-channel pipettor, add 100 μL of the revealing solution in each well.

26. Cover with parafilm and place the lid on the plate.

27. Put the plate in the incubator for 1 hour at 37 °C.

28. While the plate is in the incubator, turn on the plate reader to let it warm up.

29. Open up the plate reading program and select a file type at random (you are only reading absorbances so it really doesn't matter which one you select.

30. When the incubation time is up, remove plate from oven and place in plate reader. Read the plate using a wavelength of 405 nm.

Curriculum Vitae

EDUCATION

Bachelor of Science (Honours) in Biology (2012) Trent University, Peterborough,
Ontario, Canada.

AWARDS AND SCHOLARSHIPS

Dean's Honour Roll, Trent Univeristy, September 2008 - April 2009.

Dean's Honour Roll, Trent Univeristy, September 2010 - April 2011.

Dean's Honour Roll, Trent Univeristy, September 2011 - April 2012.

President's Honour Roll, Trent Univeristy.

TEACHING APPOINTMENTS

Teaching assistant: Bio 1002 - Biology for Science II, Western University, Winter 2014.

Teaching assistant: Bio 1001 - Biology for Science I, Western University, Fall 2013.

Teaching assistant: Bio 1002 - Biology for Science II, Western University, Winter 2013.

Teaching assistant: Bio 2244 - Statistics for Science, Western University, Fall 2012.

CONTRIBUTIONS TO RESEARCH

Invited

Kelly, T. "Life as a field researcher: collecting data and interpreting it". Oral
Presentation. Port Burwell Provincial Park, Ontario, Canada, August 2014.

Kelly, T. "A day in the life of a field researcher". Oral Presentation. Queen's University
Biological Station, Ontario, Canada, May 2014.

Contributed

Kelly, T., Scott MacDougall-Shackleton, and Elizabeth MacDougall-Shackleton.
"Immune function and infection status is related to migration distance in song
sparrows (*Melospiza melodia*)". Oral Presentation. Animal Behavior Society
Conference, Princeton University, Princeton, New Jersey, USA, August 2014.

Kelly, T., Scott MacDougall-Shackleton, and Elizabeth MacDougall-Shackleton. "Song sparrows *Melospiza melodia* allocate immune function based on migratory distance and life-history stage". Poster Presentation. Western Research Forum, Western University, London, Ontario, Canada, March 2014.

Kelly, T., Scott MacDougall-Shackleton, and Elizabeth MacDougall-Shackleton. "Song sparrows *Melospiza melodia* allocate immune function based on migratory distance and life-history stage". Poster Presentation. Sustainability and Environment Research Showcase, Western University, London, Ontario, Canada, February 2014. *Honourable mention.*

Kelly, T., Scott MacDougall-Shackleton, and Elizabeth MacDougall-Shackleton. "Stable isotope analysis of migratory distance and its relationship to immune allocation in song sparrows Melospiza melodia". Poster Presentation. Biology Graduate Research Forum, Western University, London, Ontario, Canada, October 2013.

Kelly, T., and Gary Burness. "Experimental manipulation of prenatal environment in Japanese Quail and effects on offspring phenotype". Oral Presentation. Ontario Biology Day, Laurentian University, Sudbury, Ontario, Canada, March 2012.

Burness, G., **Tosha Kelly**, Deanna Moher, and Eunice Chin. "Effects of prenatal and postnatal environmental stressors on the avian phenotype". Oral Presentation. Canadian Society of Zoologists Annual Meeting, Sackville, NB, May 2012.

EMPLOYMENT

Research Assistant: Employed by Dr. Gary Burness, Trent University, Peterborough, Ontario, Canada, May – June 2012.

Research Assistant: Employed by Dr. Jim Todd, Ontario Ministry of Agriculture Food and Rural Affairs, Simcoe, Ontario, Canada, May – August 2009.